## Herpesvirus-Encoded G Protein-Coupled Receptors as Modulators of Cellular Signaling Pathways

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# Herpesvirus-Encoded G Protein-Coupled Receptors as Modulators of Cellular Signaling Pathways

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The work described in this thesis was performed at the Leiden/Amsterdam Center for Drug Research (LACDR), Faculty of Exact Sciences, Division of Medicinal Chemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands; and at the Immunology Institute, Mount Sinai School of Medicine, New York, NY 10029-6574, USA.

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# Herpesvirus-Encoded G Protein-Coupled Receptors as Modulators of Cellular Signaling Pathways

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# Aim of the thesis

Human herpesviruses (HHV) are ubiquitously present in the human population. They are usually harmless after primary infection but they all possess the ability to remain in a silent and latent stage in the infected host. However, upon malfunctioning or temporary suppression of the host immune system, herpesviruses are reactivated and lead to serious pathological conditions. In particular, HHV-5, also known as the human cytomegalovirus (HCMV), is a major disease-causing agent in immunosuppressed transplant patients and appears to be involved in the development of vascular and proliferative diseases (Soderberg-Naucler 2006). Another well-studied herpesvirus is HHV-4, commonly known as the Epstein-Barr virus (EBV), which is involved in lymphoproliferative diseases such as Hodgkin's and Burkitt's lymphomas (Kutok and Wang 2006).

Herpesviruses have employed multiple ways to persist throughout evolution and to influence the host immune system. They have integrated host genes into their genomes and modified them to their own benefit. In particular, HHV from the beta and gamma families encode viral G protein-coupled receptors (vGPCRs) that derive from human chemokine receptors. For instance, HCMV and EBV genomes contain four (US27, US28, UL33, UL78) and one (BILF1) GPCR, respectively (Rosenkilde, Smit et al. 2008). The characteristic of most of these vGPCRs is that unlike human chemokine receptors, they are able to signal in a constitutive manner. This results in the modulation of intracellular signaling pathways and has profound effects on the biological behavior of the targeted cell. A striking example of the physiological importance of vGPCRs was demonstrated with the ORF74 gene from HHV-8 (also known as Kaposi's sarcoma-associated herpesvirus, KSHV). Numerous *in vitro* and *in vivo* studies demonstrated that the expression of this viral chemokine receptor leads to cellular transformation (Bais, Santomasso et al. 1998) and the development of Kaposi's sarcoma-like lesions in ORF74-expressing transgenic mice (Yang, Chen et al. 2000). This indicates that the expression of one single vGPCR is of crucial importance in KSHV-related diseases.

The aim of this thesis was to understand the biological relevance of the HCMV-encoded chemokine receptor US28 and of the EBV-encoded vGPCR BILF1. We identified that US28 constitutive activity is implicated in HCMV-related diseases, providing US28 as a molecular link between

viral infection and subsequent pathologies. Detailed *in vitro* studies highlighted several important signaling pathways activated by US28 during pathogenesis. We also studied the physiological consequence of BILF1 expression *in vivo* and showed that the role of this vGPCR may be important during the virus life cycle rather than for the development of EBV-related diseases.

# I

# Constitutively active human herpesviruses-encoded G protein-coupled receptors: tools for immune evasion and viral diseases

Co-authored with [2]Gerold Bongers, [2]Sergio A. Lira, [1]Martine J. Smit.

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Modified from Maussang, Bongers et al, *Chemokine Receptors as Drug Targets*, 2009, In Press.

**Abstract**

Human herpesviruses (HHV) are widespread pathogens. After primary infection of usually young individuals, these viruses remain asymptomatic in a latent stage. However, these viruses are linked to the development of a broad range of life-threatening diseases, especially in immunocompromised hosts. Interestingly, the HHV genomes encode for G protein-coupled receptors (GPCRs) that are related to the family of human chemokine receptors. Upon infection, the targeted cells express viral chemokine receptors that act in a ligand-dependent and/or ligand-independent, i.e. constitutive, manner. In turn, the modification of cellular homeostasis and physiological functions by viral chemokine receptors may contribute to the progression of viral infection and to the development of HHV-associated diseases. In this review we describe the main diseases associated with infection with the different human herpesviruses and we focus on the pharmacological and physiological properties of their respective viral GPCRs.

## Introduction

Herpesviruses are large double-stranded DNA viruses with an icosahedral nucleocapsid surrounded by a tegument and the outer lipid envelope that contains multiple viral glycoproteins. The human *herpesviriadae* family emcompasses eight members classified into 3 different subfamilies; α, β and γ. These subfamilies were established according to genetic variations in the conserved gH structural protein and possess characteristic physiological properties (Table 1). The alpha human herpesviruses (α-HHV) subfamily consists of HHV-1, HHV-2 and HHV-3, also known as herpes simplex virus (HSV)-1, HSV-2 and varicella zoster virus, respectively. They are neurotropic and cause fever blisters, genital sores and chicken pox and shingles respectively. The β-HHV subfamily is categorized into the *Cytomegalovirus* (HHV-5) and the *Roseolovirus* genus (HHV-6 and HHV-7). These viruses exhibit a broad cellular tropism and are involved in a wide range of diseases. The γ-HHV subfamiliy comprises the *lymphocryptovirus* HHV-4 and the *rhadinovirus* HHV-8. While the involvement of β-HHV in pathological conditions is becoming apparent and requires further investigation, the role of γ-HHV in oncogenesis is clearly established. These lymphotropic viruses are extensively linked to proliferative diseases due to their transforming potential.

Infections by HHV are usually asymptomatic. In order to survive within the infected immunocompetent host, viruses have acquired genes that allow them to evade the immune system or to be controlled by immune cells without being eliminated. However, upon immunosuppression, viruses escape immune surveillance and can be reactivated, leading to the development of serious pathologies. During evolution, the β- and γ-HHV have pirated genes from the human chemokine receptor family (Table 2). Although their human counterparts require ligands in order to signal and induce intracellular events, most of the viral chemokine receptors can signal in a constitutive, i.e. ligand-independent, manner. Upon viral infection, these viral receptors can perturb intracellular signaling pathways and have multiple (patho)physiological functions or be involved in the virus life cycle (Table 3).

# I. Herpesviruses and viral diseases

## The Betaherpesviridae subfamily

The cytomegalovirus genus: HHV-5/HCMV

The β-herpesvirus HHV-5, also known as the human cytomegalovirus (HCMV), is present in a wide proportion of the population. Depending on the geographical location, infection rates vary between 30% up to 100% (Britt 2008). Primary infection usually occurs within the first two decades of life and may also be congenital (Gandhi and Khanna 2004; Pereira, Maidji et al. 2005). HCMV uses both the epidermal growth factor receptor (EGFR) and the αvβ3 integrin as cellular receptors to infect a wide range of cells (Wang, Huang et al. 2005). Recently, the human platelet-derived receptor alpha (PDGFR-α) has also been shown to be important for HCMV viral entry and signaling (Soroceanu, Akhavan et al. 2008). Primary infection is usually asymptomatic and occurs in various organs such as the liver, intestine, lungs, retina and the brain. Full lytic viral replication can be observed in an exceptionally great range of cell types such as endothelial, epithelial, fibroblast, neuronal, stromal and smooth muscle (Sinzger, Digel et al. 2008). In contrast, the establishment of latent infection is restricted to specific cellular subpopulations. HCMV latent gene expression is only found in CD14+ monocytes populations, both undifferentiated and differentiated (i.e. macrophages and dendritic cells) (Reeves and Sinclair 2008). Cell types that do not support either latent or full lytic infections are polymorphonuclear cells and lymphocytes (Reeves and Sinclair 2008; Sinzger, Digel et al. 2008).

HCMV is the largest known human herpesvirus with an approximately 230 kbp double-stranded linear DNA genome (Gandhi and Khanna 2004). Several laboratory and clinical strains of HCMV have been annotated. The AD169 laboratory strain was initially predicted to encode 208 ORFs (Chee, Bankier et al. 1990), while later comparison of several clinical strains showed that they possessed 252 common ORFs (Murphy, Yu et al. 2003). However, a recent analysis comparing clinical strains to chimpanzee CMV tried to elucidate how many ORF were the most likely to encode functionally relevant proteins (Murphy and Shenk 2008). Of these, 232 ORFs were postulated to encode functional proteins involved in different functions during viral replication. Furthermore, the genetic variations between the clinical strains could relate to their different tropisms and future analysis may reveal associations of specific ORFs to HCMV-related diseases to specific strains (Murphy, Yu et al. 2003; Murphy and Shenk 2008).

The impact of HCMV infection is critical in immunocompromised patients and both acute and chronic infections play a role in viral infectious diseases. A typical case of acute infection occurs during gestation where infected fetuses can present severe hear loss or development abnormalities (Britt 2008). Another important effect of acute infection occurs during organ transplantations. Immunosuppressed transplantation patients experiencing primary HCMV infection are at stake with graft rejection and diseases specific to the engrafted organ. For instance, liver and pancreas transplant patients are prone to develop hepatitis and pancreatitis, respectively (Britt 2008). In addition, chronic HCMV infection in immunosuppressed transplant patients is known to be a cofactor for vascular diseases that result in allograft loss. The development of HCMV-associated cardiac allograft vasculopathy (CAV) commonly leads to cardiac allograft loss. Similar examples of organ-specific diseases occur in liver or kidney transplant recipients (Britt 2008). HCMV also has a major impact in human immunodeficiency virus (HIV)-infected patients. Immunodeficient patients presenting aquired immunodeficience syndrome (AIDS) are at risk with the development of HCMV-related end-organ diseases, particularly in the gastrointestinal tract and the eye. In most cases, HCMV infection leads to the development of colitis and retinitis that can eventually result in loss of vision.

Yet, also immunocompetent patients appear to be affected by HCMV infection. A recent study by Rafaidis et al. showed that patients apparently immunocompetent may be prone to the development of diseases similar to those observed in immunocompromised patients (Rafailidis, Mourtzoukou et al. 2008). Moreover, HCMV infection in immunocompetent hosts is increasingly linked to the development of inflammatory conditions such as cardiovascular and proliferative diseases (Soderberg-Naucler 2006; Britt 2008; Soderberg-Naucler 2008). For instance, HCMV is becoming a widely accepted pathogen involved in the development of atherosclerosis. It is hypothesized that HCMV can interplay with other factors regulated during this pathological condition (Soderberg-Naucler 2006). Furthermore, studies highlighted the presence of HCMV gene products in several malignancies, e.g. breast, colon and prostate cancer as well as glioblastoma (reviewed in (Soderberg-Naucler 2006; Soderberg-Naucler 2008)). Although it is important to realize that detection of HCMV products in human tissues does not necessarily reflect an association with the disease, it is clear that inflammatory conditions leads to reactivation of latent HCMV infection that can in turn maintain and/or exacerbate inflammation. In addition, HCMV is not presented as an oncogenic virus but rather as a virus that possesses oncomodulatory properties (Cinatl, Vogel et al.

2004). Cellular transformation and genetic instability may be a prerequisite for the virus to contribute to tumor progression. HCMV preferably infects tumor cells and can alter several molecular mechanisms to influence cell cycle progression, apoptosis and migration of cells. Cancer cells may also help the virus to evade immune surveillance (Michaelis, Doerr et al. 2009). As such, HCMV may further increase the transformed potential of the tumor cells while avoiding its clearance by the immune system.

The rhesolovirus genus: HHV-6 and HHV-7

HHV-6 and HHV-7 from the *Roseolovirus* genus present a high distribution worldwide with infection rates of respectively 70-100% and 75% (Safronetz, Humar et al. 2003; De Bolle, Naesens et al. 2005). Infection with HHV-6 usually occurs at an early age post-birth, around 6-9 months, while HHV-7 infection has been noted later between 1 and 2 years of age (Caselli and Di Luca 2007). There are two different variants of HHV-6, namely HHV-6A and HHV-6B, which differ in their genetic, immunological and biological characteristics (Ablashi, Balachandran et al. 1991). HHV-6 interacts with the cellular receptor CD46 to infect a wide range of cells from different origins (endothelial and epithelial cells, fibroblasts, T lymphocytes) and to target many tissues such as the endothelium, the brain, liver, tonsils and salivary glands (De Bolle, Naesens et al. 2005). HHV-6 establishes latency in monocytes and bone marrow progenitors (De Bolle, Naesens et al. 2005), but other sites such as salivary glands and the central nervous system may be involved in prolonged latency of HHV-6 (Miyake, Yoshikawa et al. 2006). HHV-7 binds to the CD4 receptor to infect primarily T lymphocytes where it also establishes a latent phase of infection (Lusso, Secchiero et al. 1994). However, it can also interact with cell surface proteoglycans, explaining the tropism of HHV-7 to cells other than T cells (Secchiero, Sun et al. 1997). HHV-6 and HHV-7 have a genome size of 160-162 kbp and 145-153 kbp encoding 110-119 open reading frames (ORF) and 84 ORFs, respectively (Caselli and Di Luca 2007).

Primary infection with HHV-6 can result in the development of exanthema subitum in a subset of children, and HHV-6B is known to be the causative agent. Complications of primary HHV-6 infection in children range from malaise, febrile seizures to more serious, but rare, conditions such as encephalitis (De Bolle, Naesens et al. 2005). HHV-6 is heavily associated with diseases of the central nervous system in immunocompromised patients such as encephalitis and/or encephalopathy (De Bolle, Naesens et al. 2005). HHV-6 is one of the

herpesvirus which is most strongly associated with multiple sclerosis. In particular, anti-HHV-6 antibodies and HHV-6 DNA have been detected in MS cerebrospinal fluid and MS plaques, respectively (Christensen 2007). So far, the link of HHV-7 and diseases other than exanthema subitum during primary infection remains highly speculative (Caselli and Di Luca 2007; Christensen 2007; Ponti, Bergallo et al. 2008). Additional studies are required to understand the true contribution of HHV-7 in pathological conditions.

## The Gammaherpesviridae subfamily

The lymphocryptovirus genus: HHV-4/EBV

The Epstein-Barr virus, discovered in 1964 (Epstein, Achong et al. 1964), is the fourth virus in the human herpesvirus family. It belongs to the genus of the lymphocryptoviruses and possesses a linear double-strand DNA of 184 kbp that encodes approximately 84 ORFs (Baer, Bankier et al. 1984). EBV is widely spread in the world population and infection is as high as 90% (Macsween and Crawford 2003). The canonical pathway for EBV infection and cell penetration in B lymphocytes and epithelial cells is mediated by the CD21 receptor (Fingeroth, Weis et al. 1984; Birkenbach, Tong et al. 1992). In addition, it has been shown that HLA Class II molecules act as coreceptor for EBV to infect B cells, and that $\beta 1$ or $\alpha 5\beta 1$ integrins can mediate CD21-independent infection in epithelial cells (Li, Spriggs et al. 1997; Tugizov, Berline et al. 2003). Primary infection usually occurs in infants at an early age or in adolescents where it can lead the development of self-resorbing mononucleosis, or kissing disease (Macsween and Crawford 2003). After control of EBV infection by the immune system, the virus remains in a latent stage in B lymphocytes and epithelial cells. During this stage, a restricted number of viral genes is expressed and latent genes are responsible for the oncogenic character of the virus (Young and Rickinson 2004). EBV is involved in a broad spectrum of diseases, which can differ between immunocompetent and immunocompromised hosts. Healthy individuals infected with EBV are more prone to develop malignancies such as Burkitt's lymphoma, Hodgkin's lymphoma and nasopharyngeal carcinoma. However, EBV is not the only factor involved in lymphoproliferative pathogenesis because not all EBV-infected individuals develop these diseases (Kutok and Wang 2006). For instance, Burkitt's lymphomas are characterized by c-myc translocations and the omnipresence of EBV seems to simply provide a biological advantage to the tumor cell.

| Subfamily | Genus | Common name | Species | Factors for viral entry | Latency sites | Genomic characteristics | Prevalence in populations | Associated diseases |
|---|---|---|---|---|---|---|---|---|
| Betaherpesvirinae | Cytomegalovirus | Human cytomegalovirus (HCMV) | HHV-5 | Epidermal growth factor, integrin αvβ3, platelet-derived growth factor receptor α | Monocytes, macrophages and dendritic cells | 230 kb, encoding between 200 and 250 ORFs | 30-100% | Immunocompromised fetus: hear loss and abnormal development<br>Immunosupressed graft recipient: graft rejection and loss, graft-specific diseases e.g. pancreatitis and hepatitis, cardiovascular diseases<br>AIDS patients: end-organ diseases, colitis and retinitis<br>Immunocompetent hosts: cardiovascular (atherosclerosis) and proliferative (colon cancer) diseases |
| | Roseolovirus | | HHV-6 | CD4, cell surface proteoglycans | Monocytes and bone marrow progenitors | 160-162 kb encoding 110-119 ORFs | 70-100% | Exanthema subitum in children<br>CNS diseases such as encephalitis, encephalopathy and multiple sclerosis |
| | Roseolovirus | | HHV-7 | CD4 | T cells | 145-153 kb encoding 84 ORFs | Approximately 75% | Exanthema subitum in children |
| Gammaherpesvirinae | Lymphocrypto-virus | Epstein-Barr virus (EBV) | HHV-4 | CD21, HLA Class II and β1 or α5β1 integrins | B lymphocytes, epithelial cells | 184 kb encoding 84 ORFs | Over 90% | Mononucleosis in adolescents (kissing disease)<br>Immunocompetent hosts: Burkitt's and Hodgkin's lymphomas, nasopharyngeal carcinoma<br>CNS diseases such as multiple sclerosis<br>Other proliferative diseases, e.g. gastric carcinoma, breast cancer, T cell lymphoma and follicular dendritic cell tumors<br>Immunosuppressed graft recipients: post-transplant lymphoproliferative disease.<br>AIDS patients: Burkitt's and Hodgkin's lymphomas, CNS lymphomas, leiosarcomas and oral hairy leukoplakia. |
| | Rhadinovirus | Kaposi's sarco-ma associated herpesvirus (KSHV) | HHV-8 | α3β1 or α5β3 integrins, DC-SIGN | B lymphocytes, epithelial cells | Approximately 170 kb encoding around 90 ORFs | 2-50% | Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease |

**Table 1.** Classification and diseases association of beta- and gamma-herpesviruses

Similarly, Hodgkin's lymphomas derives from germinal center B cells with low affinity Ig that escaped apoptosis, and the presence of EBV may provide the help necessary to rescue cells from apoptosis (Kutok and Wang 2006). Furthermore, EBV presence has been shown in other proliferative diseases, e.g. gastric carcinoma (Fukayama, Hino et al. 2008), follicular dendritic cell tumors (Shek, Ho et al. 1996), T cell lymphomas (Kutok and Wang 2006). Breast cancer may also be epidemiologically linked to EBV infection, however further investigation is required to ensure an epidemiological link (Amarante and Watanabe 2008). Finally, recent attention has been drawn on the possible association between EBV infection and multiple sclerosis (Christensen 2006). Immunocompromised hosts are also at risk with EBV-related diseases due to the reactivation of latent viruses. In particular, transplant patients undergoing immunosuppression therapies fail to have a tight regulation of EBV-infected B cells and can develop post-transplant lymphoproliferative diseases (PTLD) (Dolcetti 2007). However, PTLD can be reversed upon restoration of the immune system. In addition, HIV-infected patients in terminal AIDS phase may present EBV-related lymphomas such as Burkitt's and Hodgkin's types and lymphomas in the central nervous system (Kutok and Wang 2006). Lytic viral replication is also responsible for oral hairy leukoplakia, a non-malignant proliferative disease common in HIV patients (Walling, Flaitz et al. 2003).

The rhadinovirus genus: HHV-8/KSHV

The eighth herpesvirus was discovered in Kaposi's sarcoma lesions and named accordingly Kaposi's sarcoma herpes-virus (KSHV) (Chang, Cesarman et al. 1994). KSHV presents a double-strand genome of approximately 170 kb (Arvanitakis, Mesri et al. 1996) and encodes around 90 ORFs (Neipel, Albrecht et al. 1997). KSHV genome has a size varying from 160-170 kb (Arvanitakis, Mesri et al. 1996) up to 270 kb (Mesri, Cesarman et al. 1996; Moore, Gao et al. 1996), depending on the biological material used for sequencing, i.e. virion particles and infected cell lines respectively. KSHV infection rates are lower than other herpesviruses, ranging from a few percent up to 50% in different geographical areas (Antman and Chang 2000). Primary infection is not restricted to patients at a specific age but similarly to other herpesviruses, KHSV establishes latency in the infected host. KSHV infects cells from epithelial and endothelial origin (Kaleeba and Berger 2006) via interaction with integrins, such as the $\alpha 3\beta 1$ or $\alpha 5\beta 3$ integrins (Akula, Pramod et al. 2002; Garrigues, Rubinchikova et al. 2008). In addition, heparan sulfates play a role in KSHV penetration. B cells,

normally poorly permissive to KSHV, can be efficiently infected after overexpression of heparin sulfates (Jarousse, Chandran et al. 2008). *In vitro*, macrophages and dendritic cells are also permissive to KSHV via dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN; CD209) (Rappocciolo, Jenkins et al. 2006).

HHV-8 DNA was initially detected in Kaposi's sarcoma lesions and later on in primary effusion lymphoma (PEL) (Cesarman, Chang et al. 1995) and in multicentric Castleman's disease (MCD) (Soulier, Grollet et al. 1995). Furthermore, expression of latent viral proteins were confirmed in KS, PEL and MCD tissues, confirming the pathogenic role of KSHV in these diseases (Dupin, Fisher et al. 1999; Neipel and Fleckenstein 1999). KS, PEL and MCD have been widely reported in AIDS patients and it then became clear that the high disease prevalence in this patient population was not coincidental (Sullivan, Pantanowitz et al. 2008). HHV-8 present in HIV-infected hosts is reactivated upon the development of AIDS that in turn leads to the development of these proliferative pathologies. Failure of immune surveillance appears to be a necessary factor for the development of KSHV-related diseases since immunosuppressed transplant patients present diseases similar to those observed in AIDS patients (Ablashi, Chatlynne et al. 2002). HHV-8 has also been suggested to be associated with other diseases such as salivary gland tumors, lymphoproliferative diseases (Ablashi, Chatlynne et al. 2002) and even multiple sclerosis (Christensen 2007). However, additional studies are required to support an epidemiological link with KSHV infection.

## II. Herpesviruses encode constitutively active viral chemokine receptors

### Human cytomegalovirus encodes four different viral G protein-coupled receptors

The chemokine receptor US28 mediates HCMV-induced (patho)physiological effects

HCMV encodes four different viral GPCRs (vGPCRs), namely US27, US28, UL33 and UL78, of which US28 is the best-characterized (Chee, Satchwell et al. 1990; Gompels, Nicholas et al. 1995). Numerous publications have contributed to understand the importance of this vGPCR in a wide range of pathogenic processes. US28 protein is present on HCMV virions (Penfold, Schmidt et al. 2003) and has been found

dispensable for viral growth in HFF infected cells (Vieira, Schall et al. 1998). Recently, US28 was shown to activate the major immediate early promoter of HCMV suggesting to subsequently transactivate other viral genes (Boomker, The et al. 2006). US28 gene product is transcribed just after IE genes and before pp65 in HCMV-infected cells *in vitro* (Zipeto, Bodaghi et al. 1999). In addition to this early phase of expression, US28 mRNA and protein expressions have been determined at later time points. Various groups used quantitative PCR analysis to determine US28 translation in fully HCMV-permissive and partially-permissive cells, such as human foreskin fibroblasts, glioblastoma cell line U373MG, differentiated THP-1 cells (Zipeto, Bodaghi et al. 1999; Beisser, Laurent et al. 2001) and undifferentiated THP-1 cells respectively (Beisser, Laurent et al. 2001). Also, both latent (THP-1 cells) and fully lytic (HUVEC and HFF cells) HCMV infections leads to the transcription of US28 gene (Billstrom, Johnson et al. 1998; Beisser, Laurent et al. 2001). Finally, US28 protein expression has been widely assessed *in vitro* in HCMV-infected cells by means of radioactive chemokine binding studies (Vieira, Schall et al. 1998; Billstrom, Lehman et al. 1999; Casarosa, Menge et al. 2003; Stropes and Miller 2008).

Interestingly, US28 expression was also found *in vivo* in HCMV-infected patients. For instance, US28 mRNA expression was measured in blood samples of naturally infected hosts (Patterson, Landay et al. 1998), HIV patients (Goffard, Gault et al. 2006) as well as immunosuppressed lung transplant patients (Boomker, Verschuuren et al. 2006). US28 was also detected in neonates and fetuses presenting a congenital HCMV infection (Arav-Boger, Willoughby et al. 2002). Finally, it was recently discovered that serum of artery-coronary patients contain antibodies that recognize US28 protein, implying that US28 is or has been expressed in these patients (Bason, Corrocher et al. 2003). The primary function US28 is to bind a broad range of chemokines. US28 presents highest homology to CC and CX3C chemokine receptors (Rosenkilde, Smit et al. 2008), and binds different classes of CC and CX3C chemokines including CCL2, CCL3, CCL4, CCL5, CCL7, CCL11, CCL13, CCL26, CCL28 and CX3CL1 (Gao and Murphy 1994; Billstrom, Johnson et al. 1998; Kledal, Rosenkilde et al. 1998), as well as the KSHV-encoded viral chemokine vCXCL2 (Kledal, Rosenkilde et al. 1997) (Figure 1).

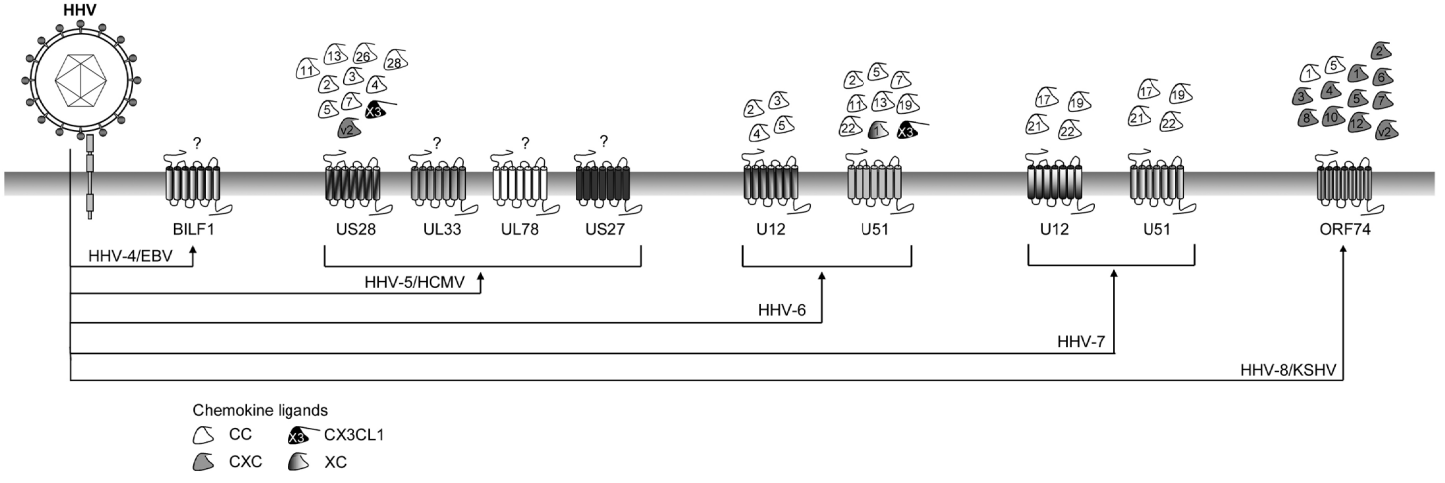


**Figure 1.** HHV-encoded vGPCRs. HHV infect cells by binding to specific cellular receptors. Upon transcription of the viral genome, vGPCRs are expressed and can bind various chemokines. Human chemokine ligands from the CC (empty), CXC (grey), CX3C (black) and XC (shadow) classes, as well as the KSHV-encoded CXCL2 chemokine (grey, v2) bind to various vGPCRs. For some receptors, ligands still need to be determined. Adapted from (Maussang, Vischer et al. 2009).

We have shown that chemokines bind to US28 by interacting with the receptor N-terminus. Point mutation analysis highlighted different amino acids involved in the interaction with either CC or CX3C chemokines (Figure 2). In particular, residues 12 and 14 appeared to mediate binding of CC chemokines, e.g. CCL3, CCL4 and CCL5, while residue 16 is specifically involved in CX3CL1 binding to US28 (Casarosa, Menge et al. 2003; Casarosa, Waldhoer et al. 2005).

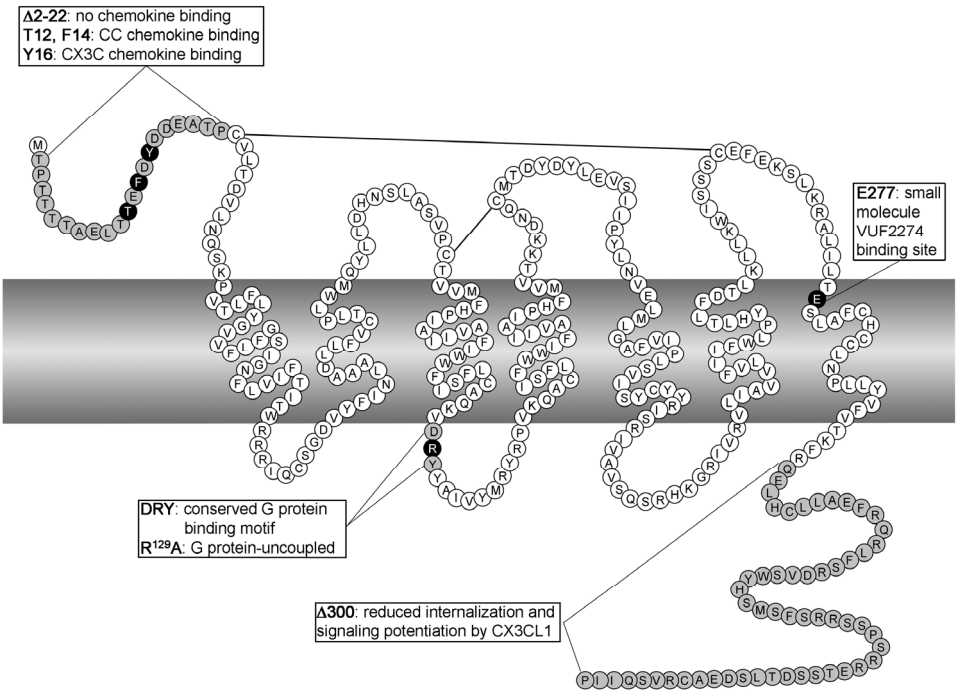


**Figure 2.** Snake-plot of US28 protein. The N-terminus and C-terminus deletion mutants of US28 are shown in grey, along with the G protein-binding DRY motif conserved in GPCR. Point mutations of US28 are highlighted in black and served to determine what residues are important for interaction with ligands and G proteins.

*Ligand-dependent US28 signaling activities*

Initially, chemokines were shown to induce calcium signaling in US28-transfected cells (CCL2 (Gao and Murphy 1994), CCL5 (Gao and Murphy 1994; Billstrom, Johnson et al. 1998), CCL7 (Billstrom, Johnson et al. 1998)), but they appear to possess different and specific signaling capacities. Several studies have demonstrated that US28 is responsible for chemokine depletion in the supernatant of HCMV-infected cells. As

such the chemokine sink function of US28 provides means for the virus to escape immune surveillance (Billstrom, Johnson et al. 1998; Bodaghi, Jones et al. 1998; Vieira, Schall et al. 1998; Billstrom, Lehman et al. 1999; Randolph-Habecker, Rahill et al. 2002).

Exposure of US28-transfected cells to CCL5 induces calcium signaling and ERK activation via $G\alpha_i$ and $G\alpha_{16}$ proteins, while CCL7 only uses $G\alpha_{16}$ proteins to modulate calcium flux (Billstrom, Johnson et al. 1998). Also, CCL2 induces calcium signaling in US28-expressing cells, but the coupling G protein still remains undetermined (Gao and Murphy 1994). Although CCL3 and CCL4 bind to US28 with high affinity, these chemokines have not been shown to induce any signaling so far (Gao and Murphy 1994; Kuhn, Beall et al. 1995). Interestingly, the modulation of calcium influx in HCMV-infected cells has also been observed after stimulation with CCL5 (Billstrom, Johnson et al. 1998). Vieira *et al.* disrupted the US28 gene from the HCMV genome by replacing it with an unrelated non-signaling gene (e.g. *Escherichia coli gpt* gene or the green fluorescence gene), and demonstrated that CCL5 stimulation in HCMV-infected cells induces calcium signaling via US28 (Vieira, Schall et al. 1998).

One of the key features of human chemokine receptors is their ability to induce chemotaxis. As such, the ability of US28 to induce cellular migration was investigated. CC chemokines CCL2 and CCL5 were shown to induce US28-mediated chemotaxis via $G\alpha_{12}$ rather than $G\alpha_{i/o}$ proteins (Streblow, Soderberg-Naucler et al. 1999; Melnychuk, Streblow et al. 2004). Treatment of US28-expressing smooth muscle cells (SMC) with pertussis toxin showed no inhibitory effects of CCL2 or CCL5-induced chemotaxis (Streblow, Soderberg-Naucler et al. 1999), while co-transfection of $G\alpha_{12}$ or $G\alpha_{13}$ proteins enhanced US28-mediated migration of HCMV-infected cells (Melnychuk, Streblow et al. 2004; Vomaske, Melnychuk et al. 2009). In addition, $G\alpha_{12}$ cotransfection potentiated US28-activated focal adhesion kinase (FAK) and RhoA kinase signaling (Melnychuk, Streblow et al. 2004; Vomaske, Melnychuk et al. 2009). Recently, it was shown that CX3CL1 induces the chemotaxis of macrophages but not of SMC. CX3CL1 antagonizes CCL5-induced SMC chemotaxis and induces FAK activation via $G\alpha_q$ proteins (Vomaske, Melnychuk et al. 2009). In SMC, US28 was also shown to induce p42/p44 MAPK activation upon stimulation with CCL5 or CX3CL1 (Vomaske, Melnychuk et al. 2009). Also in a viral context, US28 is responsible for HCMV-induced cell migration. The deletion of the US28 gene from the HCMV viral genome impairs cellular migration observed in infected cells (Streblow, Soderberg-Naucler et al. 1999) and the cotransfection of either $G\alpha_{12}$ or $G\alpha_{13}$ further increases the migration of HCMV-infected cells (Melnychuk, Streblow et al. 2004). Taken together,

these data present US28 as a molecular mediator of atherosclerosis pathogenesis and viral dissemination in HCMV-infected patients.

*Ligand-independent US28 signaling*

Besides the ligand-induced activities observed in US28-expressing cells, this vGPCR possesses prominent ligand-independent activity. We initially demonstrated that expression of US28 induces the constitutive activation of the phospholipase C and the transcription factor NF-κB (Casarosa, Bakker et al. 2001). This constitutive signaling involves $G\alpha_q$ and $G\beta\gamma$ subunits and pre-treatment of the cells with pertussis toxin confirmed that these cellular processes were not mediated by $G\alpha_{i/o}$ proteins. Using a G protein-uncoupled mutant of US28, US28-$R^{129}$A, and a US28 mutant that cannot bind ligands, US28-Δ2-22, we demonstrated that G protein activation, but not chemokine binding, were important for the constitutive formation of inositol by US28 (Casarosa, Menge et al. 2003; Waldhoer, Casarosa et al. 2003) (Figure 2). US28 is also responsible for the activation of the cyclic AMP responsive element binding protein (CREB) and the nuclear factor activated in T cells (NFAT) transcription factors through p38 MAPK- and p42/p44 MAPK–dependent pathways (McLean, Holst et al. 2004). Pre-treatment of cells with PTX indicated that NFAT transcriptional activation partly relied on a $G\alpha_i$ component (McLean, Holst et al. 2004). Recently, the constitutive activation of $G\alpha_q$ subunits has also been shown to induce the transcription of the serum responsive element (SRE) (Moepps, Tulone et al. 2008). When cotransfecting $G\alpha_{16}$ subunits into US28-expressing cells, SRE activation was impaired, suggesting that instead of being constitutively activated by US28, $G\alpha_{16}$ competes with $G\alpha_q$ during receptor coupling (Moepps, Tulone et al. 2008). As such, signaling studies indicate that US28 couples to a broad range of G proteins, such as $G\alpha_{i/o}$, $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ subunits upon ligand stimulation, whereas US28 constitutive activity is mediated mainly by $G\alpha_{q/11}$ and $G\beta\gamma$ proteins.

In order to respond to chemokine stimulation, human chemokine receptors are expressed at the cell surface where they can bind their respective ligands present in their microenvironment. Upon ligand binding, chemokine receptors elicit activation of intracellular signaling cascades and further undergo internalization before being degraded or recycled to the cell surface (Neel, Schutyser et al. 2005). The viral chemokine receptor US28 is able to undergo internalization independently from ligand stimulation (Fraile-Ramos, Kledal et al. 2001). Transfection of US28 resulted in merely 20% of cell surface expression and most of intracellular expression was located in multivesicular endosomes. Interestingly, the use of the G protein-

uncoupled mutant of US28, US28-$R^{129}A$, revealed that constitutive internalization was independent from constitutive G protein-mediated signaling activity (Fraile-Ramos, Kledal et al. 2001). In contrast, the C-terminal tail of US28 showed to be the crucial motif responsible for the ligand-independent endocytosis of the receptor (Waldhoer, Casarosa et al. 2003). CX3CL1 is the only chemokine able to downmodulate US28 constitutive activity in several signaling assays, and it was as such classified as an inverse agonist (Casarosa, Bakker et al. 2001; Waldhoer, Kledal et al. 2002; McLean, Holst et al. 2004). However, when US28 internalization was impaired by removing its C-terminal tail or replacing it by that of another human or viral GPCR, the receptor presented a higher signaling activity than the non-mutated receptor and CX3CL1 could further potentiate its signaling (Waldhoer, Casarosa et al. 2003). As such, the constitutive internalization of US28 affects its signaling ability and it was suggested to mask the true agonist properties of its ligands.

US28 constitutive activity may also have an impact on the signaling abilities of other ligand-stimulated chemokine receptors. We showed that although CCL5 is a ligand for both US28 and CCR1, it failed to stimulate US28 constitutive activity or CCR1 signaling towards NF-κB (Bakker, Casarosa et al. 2004). Cotransfection of CCR1 without chemokine stimulation in US28-expressing cells also did not potentiate constitutive signaling. However, CCL5 stimulation was able to increase NF-κB activation when both CCR1 and US28 were coexpressed (Bakker, Casarosa et al. 2004). Interestingly, the use of the US28-Δ2-22 and US28-$R^{129}A$ mutants indicated that CCL5 was not mediating its potentiating effect via US28 but directly via CCR1, and that the constitutive activity of US28 was required to observe NF-κB signaling, respectively. This potentiation was entirely PTX-sensitive, demonstrating that ligand stimulation induced the coupling of CCR1 to $G\alpha_{i/o}$ proteins. As such, ligand-stimulated $G\alpha_{i/o}$ protein coupling can be diverted by US28 to enhance its own constitutive signaling.

The constitutive activity of US28 was shown in several studies in a viral setting. Deletion of the US28 gene from the genome of laboratory (AD169 and Toledo) and clinical (TB40/E) HCMV strains showed that this vGPCR is the main mediator of the constitutive activation of $G\alpha_q$-mediated signaling pathways in HCMV-infected human foreskin fibroblast cells (Minisini, Tulone et al. 2003). In addition, US28 ligand-independent activity has been shown to be responsible for the signaling observed in virally infected cells. Mutant viruses presenting a deletion of the N-terminus of US28 could induce inositol phosphate formation at a similar level than WT HCMV strains, indicating that chemokine binding

did not play a role in the observed PLC activation (Stropes and Miller 2008).

In order to block US28 constitutive activity, we have developed a series of compound that can act as inverse agonists. The first nonpeptidergic compound, VUF2274, was derived from an antagonist against the chemokine receptor CCR1 (Casarosa, Menge et al. 2003). Besides inhibiting US28-mediated signaling activity in transfected cells, VUF2274 could also reduce the constitutive activity of US28 observed in HCMV-infected cells (Casarosa, Menge et al. 2003). Mutation analysis of a glutamic acid residue in the seventh transmembrane domain into an alanin residue (E277A) showed that this site was partly involved in the interaction of the VUF2274 compound with US28. Such inverse agonist compounds may be promising anti-viral drugs, but further optimization and improvement of the affinity of these compounds is still required (Hulshof, Casarosa et al. 2005; Hulshof, Vischer et al. 2006).

*Potential roles of US28 in HCMV-related pathological conditions*

A role for US28 in cardiovascular diseases was demonstrated by Streblow *et al.* (Streblow, Soderberg-Naucler et al. 1999). Using vascular smooth muscle cells, they showed that the deletion of US28 from the HCMV genome impaired the cellular migration induced after viral infection. In addition, expression of US28 in vascular smooth muscle cells induced chemotaxis towards CCL2 and CCL5, providing a molecular link between HCMV infection and the development of cardiovascular diseases (Table 2). After CCL5 binding, US28 activates $G\alpha_{12}$ proteins and its downstream effector RhoA kinase (Melnychuk, Streblow et al. 2004). Furthermore, US28-mediated cell migration in HCMV-infected cells was sensitive to protein tyrosine kinase inhibitors (Streblow, Soderberg-Naucler et al. 1999). Upon CCL5 and CX3CL1 stimulation, US28 interacts and activates Src kinase, which phosphorylates the focal adhesion kinase (FAK) and activates Grb2/Sos (Streblow, Vomaske et al. 2003; Vomaske, Melnychuk et al. 2009). Besides highlighting cell-specific phenotypes, the chemotactic responses elicited by CCL5 and CX3CL1 chemokines in SMC and macrophages, respectively, also provide several functions for US28 during the development of HCMV-related cardiovascular diseases (Vomaske, Melnychuk et al. 2009). US28 expression may on one hand induce macrophage infiltration into atherosclerotic plaques, while on the other hand, favor the recruitment of SMC to the inflamed lesion. As such, US28 expression may play an important role during the pathogenesis of cardiovascular diseases in HCMV-infected patients.

In addition, another function of US28 resides in its ability to facilitate HIV-1 entry (Table 2). Pleskoff et al. demonstrated that US28 expression in CD4+ cell lines is sufficient to allow HIV-1 entry (Pleskoff, Treboute et al. 1997). Since US28 presents homology to chemokine receptors, which have previously been to shown to act as co-receptors for HIV, US28 may possess common motifs that allow recognition by HIV and play a role in the interplay between HCMV and HIV-1 virus. The US28 inverse agonist VUF2274 is able to reduce US28-mediated HIV-1 entry (Casarosa, Menge et al. 2003), confirming US28 as a potential drug target in treatment of HIV infection.

**Table 2.** Viral GPCRs homology to human chemokine receptors (CKR) and their associated functions

| Virus | vGPCR | CKR homolog | Putated functions |
|---|---|---|---|
| HHV-4 | BILF1 | CXCR4 | Immune evasion: downregulates MHC Class I and PKC<br>Homeostasis and cellular trafficking: heterodimerizes with a variety of human chemokine receptors in B cells |
| HHV-5 | US28 | CX3CR1 and CCR5 | Atherosclerosis: mediates smooth muscle cells migration in HCMV-infected cells and transfected cells<br>HIV infection: mediates HIV-1 entry |
| | UL33 | CCR10 | Viral life cycle: Murine and rat orthologs important for viral replication in salivary glands and cellular migration |
| | UL78 | CXCR1 | Viral life cycle: murine and rat orthologs important for virulence and viral |
| | US27 | CXCR3 | Unknown |
| HHV-6 | U12 | CCR10 | Unknown |
| | U51 | - | Immune evasion:downregulates CCL5 and FOG-2<br>Viral life cycle: important for viral replication, induction of cytopathic effects and can enhance cell-cell fusion in infected cells |
| HHV-7 | U12 | CX3CR1 | Unknown |
| | U51 | CCR2 | Unknown |
| HHV-8 | ORF74 | CXCR2 | Oncogenesis: induces a pro-angiogenic and inflammatory phenotype (release of cytokines and growth factors and upregulation of adhesion molecules)<br>Oncogenesis: transforms cells in direct and paracrine manners<br>Oncogenesis: induces tumor formation in xenograft models and transgenic animals |

## The constitutively active orphan chemokine receptor UL33

UL33 RNA transcripts are expressed in the early phase of HCMV infection (Bodaghi, Jones et al. 1998) and its protein product has been found in viral particles (Margulies, Browne et al. 1996; Varnum, Streblow et al. 2004). UL33 presents highest homology to the chemokine receptor CCR10 (Vischer, Vink et al. 2006) but to date no ligands have been identified for this vGPCR. We have investigated the signaling abilities of this receptor and shown that UL33 can activate multiple G proteins in a ligand-independent manner. In COS-7 cells, expression of UL33

constitutively led to the production of inositol phosphates partially via $G\alpha_{q/11}$ and $G\alpha_{i/o}$. The modulation of CREB-driven gene transcription was mediated by the $G\alpha_{i/o}$, $G\alpha_s$ and $G\beta\gamma$ subunits (Casarosa, Gruijthuijsen et al. 2003), as well as the protein kinase p38 (Waldhoer, Kledal et al. 2002). In addition, in a viral context UL33 was partially responsible for the HCMV-induced CREB activation in infected U373 cells (Casarosa, Gruijthuijsen et al. 2003).

While the roles of UL33 during viral life cycle or virus-induced diseases still remain undefined, some light has been shed on the importance of its rodent orthologs such as the murine M33 and rat R33 receptors (Table 2). Both rodent receptors belong to the UL33 gene family (Waldhoer, Kledal et al. 2002) and similarly to UL33, both M33 and R33 were shown to be dispensable for viral replication *in vitro* (Beisser, Vink et al. 1998; Melnychuk, Smith et al. 2005). However, both the M33 and R33 receptors are important for viral replication in the salivary glands of rodent CMV-infected animals (Davis-Poynter, Lynch et al. 1997; Beisser, Vink et al. 1998). Also, M33 and R33 exhibit ligand-independent signaling towards NF-κB and CREB via $G\alpha_{q/11}$ and $G\alpha_{i/o}$ proteins (Gruijthuijsen, Casarosa et al. 2002; Waldhoer, Kledal et al. 2002). In contrast, the rodent orthologs present slightly different signaling abilities compared to their human counterpart UL33 (Waldhoer, Kledal et al. 2002; Casarosa, Gruijthuijsen et al. 2003). This is particularly true regarding the development of virus-associated diseases. We have discussed above the involvement of US28 in virus-induced cardiovascular diseases. In a similar manner, both R33 and M33 are important for the migration of respectively rat and mouse CMV-infected smooth muscle cells *in vitro* (Melnychuk, Smith et al. 2005; Streblow, Kreklywich et al. 2005). In a physiological *in vivo* model, RCMV infection of immunocompromised rats led to a high mortality of heart-transplanted animals compared to non-infected recipients. Infection of animals with the R33 deletion mutant of RCMV resulted in a lower mortality compared to animals infected with WT RCMV, which was due to a milder development of transplant vascular sclerosis and subsequent slower graft rejection (Streblow, Kreklywich et al. 2005). As such, the rodent UL33 genes present similar signaling properties to human UL33, but their functions and signaling abilities may compensate for the lack of US28 orthologs in these non-human viruses (Waldhoer, Kledal et al. 2002).

The chemokine receptor-related US27 and UL78 orphan receptors

The US27 and UL78 HCMV-encoded GPCRs are the least characterized. US27 is considered a chemokine receptor presenting 23% homology to

CXCR3 (Vischer, Vink et al. 2006) but so far neither chemokine ligands (Bodaghi, Jones et al. 1998) nor putative functions in viral life cycle or related pathologies have been identified. In HCMV-infected cells, US27 is expressed during the late phase of infection and its protein product is heavily glycosylated (Bodaghi, Jones et al. 1998; Vieira, Schall et al. 1998; Margulies and Gibson 2007). Furthermore, several reports indicate that US27 protein is present in HCMV virions (Fraile-Ramos, Pelchen-Matthews et al. 2002; Varnum, Streblow et al. 2004; Margulies and Gibson 2007).

UL78 presents very low homology to chemokine receptors with only 13% to CXCR1 (Vischer, Vink et al. 2006), and some other reports indicate that it may not be related to this family of GPCRs (Rosenkilde, Smit et al. 2008). Michel and colleagues have investigated the importance of UL78 in HCMV viral life cycle and characterized it as an early gene that is also transcribed in the late phase of infection (Michel, Milotic et al. 2005). Deletion of the UL78 gene from the HCMV genome resulted in an unaltered ability of the virus to replicate both in human foreskin fibroblasts as well as in a renal artery organ-culture system (Michel, Milotic et al. 2005). In contrast, the mouse M78 and rat R78 genes, which are also expressed during the early phase of infection, showed to be crucial for viral replication *in vitro* (Beisser, Grauls et al. 1999; Oliveira and Shenk 2001) and the production of infectious RCMV particles in the spleen *in vivo* (Kaptein, Beisser et al. 2003). Interestingly, deletion of the R78 gene from the RCMV genome resulted in an increased survival of RMCV-infected immunocompromised rats, highlighting an important role for R78 in the pathogenesis of RCMV *in vivo* (Beisser, Grauls et al. 1999).

### Roseoloviruses-encoded chemokine receptors: the U12 and U51 genes

U12 and U51 genes were first identified in the U1102 strain of the HHV-6A subfamily after DNA sequencing of the viral genome (Gompels, Nicholas et al. 1995) and were later functionally characterized as chemokine receptors (Isegawa, Ping et al. 1998; Milne, Mattick et al. 2000). Both chemokine receptors have different transcription kinetics in HHV-6-infected cells. U12 is expressed late during the viral replication phase (Isegawa, Ping et al. 1998), while U51 appears early after viral infection (Menotti, Mirandola et al. 1999). Homologues of the U12 and U51 genes have been found in HHV-7 (Nakano, Tadagaki et al. 2003; Tadagaki, Nakano et al. 2005), but the U12 and U51 genes from HHV-6 and HHV-7 present different chemokine binding profiles. HHV-6A U51 has been shown to bind CCL2, CCL5, CCL7, CCL11, CCL13, CCL19,

CCL22, CX3CL1 and XCL1 (Milne, Mattick et al. 2000; Catusse, Spinks et al. 2008), while HHV-7 U51 binds CCL17, CCL19, CCL21 and CCL22 but not CCL1, CCL2, CCL5 or CXCL8 (Tadagaki, Nakano et al. 2005). Similarly, U12 from HHV-6B and HHV-7 bind CCL2, CCL3, CCL4, CCL5 (Isegawa, Ping et al. 1998) and CCL17, CCL19, CCL21, CCL22 (Tadagaki, Nakano et al. 2005) respectively. Furthermore, HHV-6A U51 can constitutively modulate signaling pathways (Fitzsimons, Gompels et al. 2006). While the constitutive activation of $G\alpha_q$ proteins by U51 in transfected COS-7 cells resulted in the production of inositol phosphate and the inhibition of cAMP responsive element (CRE)-mediated signaling, CCL2, CCL5 and CCL11 specifically modulated these signaling pathways in different manners. The three chemokines could reverse the constitutive CREB inhibition, acting as inverse agonist. However, CCL5 could also further increase U51-mediated PLC activation. The different chemokines were hypothesized to bind to different active states of U51, further modulating the downstream signaling of the receptor and acting as diverse pharmacological modulators (Fitzsimons, Gompels et al. 2006). Besides binding endogenous human chemokines and affect cellular chemotactic properties, HHV-6A U51 has been shown to downregulate CCL5 and FOG-2 mRNA expressions (Milne, Mattick et al. 2000; Catusse, Spinks et al. 2008). The transcriptional repression of these immunomodulatory genes may represent a way for HHV-6 to subvert the immune system and avoid recognition by immune cells (Catusse, Spinks et al. 2008). The function of U51 during HHV-6A viral life cycle has also been studied *in vitro*. With the use of siRNA against U51, it was shown that U51 is important for viral replication and induction of cytopathic effects after HHV-6A infection (Zhen, Bradel-Tretheway et al. 2005). Furthermore, U51 can enhance cell-cell fusion but apparently not the virus-cell interaction (Zhen, Bradel-Tretheway et al. 2005). So far, the signaling pathways activated by HHV-7 U51 and U12 from both HHV-6 and -7 have not been delineated and further studies are required to understand how these receptors may affect cellular functions and viral life cycles of their respective viruses.

## EBV-encoded constitutively active orphan vGPCR BILF1

BILF1 is expressed as an early or immediate-early viral gene after EBV infection. BILF1 expression is restricted to the lytic phase of infection and is not associated to any of the latency programs (Beisser, Verzijl et al. 2005; Kutok and Wang 2006; Kaptein, Jungscheleger-Russell et al. 2008). This vGPCR is hypothesized to belong the class of chemokine receptors, presenting a low homology to CXCR4 (Vischer, Vink et al. 2006), but so far no chemokines have been found to bind to this

receptor. As such, BILF1 is still considered an orphan receptor. However, like other HHV-encoded chemokine receptors, BILF1 constitutively activates several signaling pathways. For instance, BILF1 expression has been shown to modulate CRE- and NF-κB-mediated gene activation in COS-7 and B cells via $G\alpha_{i/o}$ proteins (Beisser, Verzijl et al. 2005; Paulsen, Rosenkilde et al. 2005). Interestingly, we have recently shown that BILF1 can heterodimerize with a wide variety of chemokine receptors, which could potentially affect their normal physiological functions and affect cellular migration capacities (Vischer, Nijmeijer et al. 2008).

To this date, the functional consequence of BILF1 expression tends towards evasion from immune surveillance. Expression of BILF1 *in vitro* leads to the inhibition of phosphorylated RNA-dependent protein kinase (PKR) phosphorylation (Beisser, Verzijl et al. 2005). PKR plays an important function in cellular antiviral defense and the constitutive activity of BILF1 was postulated to help EBV to prevent the host immune response. Additionally, it was recently discovered that BILF1 can downmodulate the surface expression the MHC class I but not MHC class II proteins (Zuo, Currin et al. 2009). BILF1 was immunoprecipitated from transfected cells together with MHC class I molecules and these antigen presenting molecules were more rapidly internalized from the cell surface and subsequently degraded. In addition, overexpression of BILF1 in EBV-infected cells was able to reduce CD8+ T cell activation (Zuo, Currin et al. 2009). As such, expression of BILF1 may provide a way for the virus to escape the immune system.

### ORF74, the tumorigenic constitutively active chemokine receptor encoded by KSHV

The KSHV genome possesses a single chemokine receptor, ORF74, which was initially identified as a homologue of the IL-8 receptor (Cesarman, Nador et al. 1996; Guo, Browning et al. 1997). ORF74 binds a broad range of human CC and CXC chemokine ligands, namely CCL1, 5 and CXCL1, 2, 3, 4, 5, 6, 7, 8, 10, 12, as well as the KSHV-encoded viral chemokine vCXCL2 (Arvanitakis, Geras-Raaka et al. 1997; Geras-Raaka, Varma et al. 1998; Geras-Raaka, Varma et al. 1998; Gershengorn, Geras-Raaka et al. 1998; Rosenkilde, Kledal et al. 1999; Rosenkilde, Kledal et al. 2000). ORF74 expression is expressed during the early phase of lytic infection *in vitro* (Kirshner, Staskus et al. 1999). Its mouse ortholog has been shown to be dispensable for viral growth (Lee, Koszinowski et al. 2003). Interestingly, ORF74 can transactivate different promoters of KSHV viral genes, implying a potential regulatory function for ORF74 during viral infection (Chiou, Poole et al. 2002). In

addition, ORF74 gene products have been detected in the main KSHV-related diseases, i.e. KS, PEL and MCD (Cesarman, Nador et al. 1996; Guo, Browning et al. 1997; Cobo, Hernandez et al. 1999; Chiou, Poole et al. 2002). Initial studies on ORF74 rapidly demonstrated the oncogenic potential of this receptor (Arvanitakis, Geras-Raaka et al. 1997; Bais, Santomasso et al. 1998) and its signaling abilities were subsequently determined.

ORF74 constitutively activates multiple signaling pathways in different cell types

Initial characterization of ORF74 signaling properties demonstrated that this viral chemokine receptor signals in a constitutive manner via $G\alpha_{q/11}$ proteins. ORF74 expression leads to the constitutive formation of inositol phosphates (Arvanitakis, Geras-Raaka et al. 1997; Smit, Verzijl et al. 2002). ORF74 also triggers constitutive signaling through $G\alpha_{i/o}$ and $G\beta\gamma$ subunits (Montaner, Sodhi et al. 2001; Smit, Verzijl et al. 2002) and through $G\alpha_{13}$ proteins (Shepard, Yang et al. 2001). In addition to classical trimeric G proteins, ORF74 constitutive activity can be mediated by small G proteins such as Rac1 (Dadke, Fryer et al. 2003). ORF74-induced G protein coupling appears to be cell type-dependent, highlighting the potential of ORF74 to adapt to different cellular context to perturb cellular homeostasis. The activation of a broad range of G proteins leads to the activation of various downstream kinases possessing different functions in ORF74-induced phenotypes. For instance, the constitutive stimulation of the protein kinase C (PKC) mediated by ORF74 (Arvanitakis, Geras-Raaka et al. 1997) or the PMA-induced PKC stimulation results in an inhibition of $G\alpha_q$-mediated ORF74 constitutive signaling (Geras-Raaka, Arvanitakis et al. 1998). Similarly, co-transfection of the GPCR-specific kinases GRK4, GRK5 or GRK6 inhibits ORF74 constitutive activity by desensitizing the receptor (Geras-Raaka, Arvanitakis et al. 1998). However, kinases traditionally associated with inflammation and proliferation were shown to be activated by ORF74 in a constitutive manner. This included members of the MAPK family such as p44/42 (Sodhi, Montaner et al. 2000; Smit, Verzijl et al. 2002; Cannon, Philpott et al. 2003), p38 (Bais, Santomasso et al. 1998; Sodhi, Montaner et al. 2000; Cannon, Philpott et al. 2003) and JNK (Bais, Santomasso et al. 1998; Sodhi, Montaner et al. 2000), but also Akt (Montaner, Sodhi et al. 2001; Smit, Verzijl et al. 2002; Cannon, Philpott et al. 2003). Other kinases involved in cell cycle regulation, e.g. mTOR (Sodhi, Chaisuparat et al. 2006) and the p21-activated kinase 1 (PAK1) (Dadke, Fryer et al. 2003) are also important for ORF74-induced transformation. Different *in vitro* settings either

inhibiting or overactivating these pathways highlighted the importance of these different kinases during ORF74-induced pathogenic conditions. Similarly, ORF74 can also constitutively activate several transcription factors that have been linked to inflammation and proliferation. Initially, a link between ORF74 and angiogenesis was established because of the presence of ORF74 in highly vascularized KS lesions. ORF74-expressing cells were shown to activate the hypoxia-inducible factor 1$\alpha$ (HIF1$\alpha$) (Sodhi, Montaner et al. 2000). A wide variety of transfected cells also present a constitutive activation of different transcription factors such as AP-1, NF-$\kappa$B, CREB, NFAT and SRE (Montaner, Sodhi et al. 2001; Schwarz and Murphy 2001; Shepard, Yang et al. 2001; Cannon, Philpott et al. 2003; Pati, Foulke et al. 2003; Rosenkilde, McLean et al. 2004), which can further deregulate the expression of pro-angiogenic and inflammatory factors. A non-exhaustive list of molecules contributing to ORF74-derived pathologies includes the vascular endothelial growth factor VEGF and its receptor VEGFR2 (Bais, Van Geelen et al. 2003), the inflammatory cytokines IL2, IL-4, IL-6 and GM-CSF (Pati, Cavrois et al. 2001; Pati, Foulke et al. 2003), the chemoattractant factors CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL8 (Pati, Cavrois et al. 2001; Schwarz and Murphy 2001; Polson, Wang et al. 2002) and adhesion molecules such as VCAM-1, ICAM-1 and E-selectin (Pati, Cavrois et al. 2001) (Table 3). It is important to note that the secreted factors, along with the different modulated signaling pathways are highly cell type-dependent and highlight the ability of ORF74 to adapt to the cellular context to exert its pathogenic effects (Pati, Cavrois et al. 2001; Schwarz and Murphy 2001; Polson, Wang et al. 2002; Pati, Foulke et al. 2003).

The chemokines binding to ORF74 have all been pharmacologically characterized using G protein-coupling assays such as cAMP accumulation assays and IPx formation. Studies revealed that CXCL10, CXCL12 and vCXCL2 act as full inverse agonists; CXCL6 is a partial inverse agonist; CXCL4, 5, 7 and 8 act as neutral ligands; CXCL2 is a partial agonist; and CXCL1 and CXCL3 are full agonists (Geras-Raaka, Varma et al. 1998; Geras-Raaka, Varma et al. 1998; Gershengorn, Geras-Raaka et al. 1998; Rosenkilde, Kledal et al. 1999; Rosenkilde, Kledal et al. 2000). The two most interesting and best characterized ligands for ORF74 are CXCL1 and CXCL10. Their pharmacological behaviors have been investigated beyond the scope of G protein coupling. The respective agonist and inverse agonist activities of CXCL1 and CXCL10 were highlighted during physiological relevant mechanisms such as DNA synthesis, activation of kinases (Akt and MAPK p44/42) and the NF-$\kappa$B transcription factor, as well as in the release of CXCL8 and VEGF (Geras-Raaka, Varma et al. 1998; Schwarz and Murphy 2001; Shepard, Yang et al. 2001; Smit, Verzijl et al. 2002; Cannon, Philpott et

al. 2003). As such, these two chemokines may play prominent roles during the development of ORF74-related pathological conditions.

ORF74 expression mimics KSHV-induced transformation

Early studies indicated that the cells transformed with the KSHV-encoded vGPCR ORF74 show an increase in cell proliferation (Arvanitakis, Geras-Raaka et al. 1997). Furthermore, ORF74-expressing NIH-3T3 cells induced foci formation and injection of these cells in nude mice led to the formation of angiogenic lesions resembling KS (Bais, Santomasso et al. 1998). The strongest argument to date regarding the significance of ORF74 in inducing angiogenesis and inflammation in KS is derived from *in vivo* studies in mice. Transgenic mice expressing ORF74, either under the control of a segment of the CD2-promoter or by specifically targeting expression to endothelial cells using an *in vivo* retroviral-mediated infection system, develop angioproliferative lesions and tumors that strongly resemble those observed in KS patients (Yang, Chen et al. 2000; Montaner, Sodhi et al. 2003).

As discussed above, ORF74 is constitutively active but its activity can be enhanced by angiogenic chemokines and inhibited by angiostatic chemokines. To delinate the role of the ligand-dependent and ligand-independent ORF74-mediated angioproliferation, transgenic mice were generated carrying mutant ORF74 (Holst, Rosenkilde et al. 2001). Transgenic mice with a mutant ORF74 capable of ligand-binding and activation, but deficient in constitutive signalling, did not develop any angiogenic lesions by 90 days of age, despite cell-surface expression of this mutant receptor. To determine if chemokine binding was required for pathogenesis, a N-terminal deletion mutant of ORF74 that was unable to bind chemokines was generated (Ho, Du et al. 1999; Rosenkilde, Kledal et al. 2000). Transgenic mice carrying this N-terminal deletion mutant did not develop any KS-like tumours, despite the fact that the mutant receptor still displayed unaltered high constitutive signalling activity. Finally, transgenic expression of a mutant ORF74, which preserved high constitutive activity but did not respond to the agonist chemokines CXCL1 and its murine ortholog CXCL2, resulted in reduced penetrance and severity of the angioproliferative disease. These results indicate that modulation of the high constitutive activity of ORF74 by endogenous angiogenic chemokines is an important factor in the development of a KS-like angioproliferative disease in mice, since the constitutive activity of the receptor alone is not sufficient. The modulation of ORF74 activity within tissues is probably mediated by chemokines expressed within the KS lesions, but the mechanisms of

chemokine production and the kinetics of this interaction are presently unknown.

Both in human KS and in KS-like lesions found in ORF74-transgenic mouse models, expression of ORF74 is observed only in a subpopulation of spindle cells (Yang, Chen et al. 2000; Montaner, Sodhi et al. 2003; Guo, Pati et al. 2004). Therefore, it was suggested that the ORF74-mediated angioproliferation is due to paracrine mechanisms. As described above, ORF74-mediated signalling involves activation of e.g. VEGF, Akt-mTOR, and NF-κB, leading to the induction of proangiogenic and proinflammatory factors (Pati, Cavrois et al. 2001). Co-injection of ORF74-expressing endothelial cells with cells expressing KSHV latent genes into nude mice greatly increased the tumorigenic potential of ORF74 compared to injection of ORF74-expressing cells alone. Although non-transformed endothelial do not proliferate by themselves, the presence of ORF74 on surrounding cells, through presumably a paracrine mechanism, leads to an increased tumorigenic potential of these cells (Montaner, Sodhi et al. 2003). Furthermore, ORF74-mediated neoplasia was shown to be dependent on activation of NF-κB and Akt. In human KS, both these pathways are over-activated, indicating their importance in the pathogenesis of human KS (Sodhi, Montaner et al. 2004; Martin, Galisteo et al. 2008).

On the other hand, there is also strong evidence that ORF74, beside activating a paracrine mechanism, plays a direct role angioproliferation and tumorigenesis. This was show by using transgenic mice that express both ORF74 and β-galactosidase (LacZ) (Jensen, Manfra et al. 2005; Grisotto, Garin et al. 2006). Cells expressing ORF74 and LacZ within proliferative lesions were phenotyped, their distribution mapped in early lesions and tumors, and their relevance to angioproliferation and tumorigenesis was evaluated. The ORF74/LacZ$^{+}$ cells were found to express markers of endothelial progenitor cells; they proliferated upon transgene activation (DOX-treatment) and transferred disease to immunodeficient RAG1$^{-/-}$ mice. Furthermore, ORF74/LacZ$^{+}$ cells surrounded tumors but were scarce within them, mimicking what is observed in human KS. DNA profiling of tumor-derived laser-microdissected LacZ$^{-}$ cells suggested that they originated from the same population as vGPCR/LacZ$^{+}$ cells. These findings suggest that ORF74 may promote tumorigenesis by a hit-and-run mechanism like other oncogenes. ORF74 may induce an early autocrine component characterized by angioproliferation and inflammation followed by a second phase of tumorigenesis (Jensen, Manfra et al. 2005; Grisotto, Garin et al. 2006).

| Herpesvirus Class | β-HHV | | | γ-HHV | |
|---|---|---|---|---|---|
| **Viral chemokine receptor** | **US28** | **UL33** | **U51** | **ORF74** | **BILF1** |
| Coupling G-proteins | $\alpha_{q/11}$, $\alpha_{12/13}$, $\alpha_{16}$, $\alpha_{i/o}$, $\beta\gamma$ | $\alpha_{q/11}$, $\alpha_{i/o}$, $\beta\gamma$ | $\alpha_{q/11}$ | $\alpha_{q/11}$, $\alpha_{13}$, $\alpha_{i/o}$, $\beta\gamma$ | $\alpha_{i/o}$ |
| Activated down-stream kinases | MAPK p44/42, MAPK p38, FAK, RhoA | MAPK p38 | ? | MAPK p44/42, MAPK p38, MAPK JNK, PKB, PKC, JAK2, mTOR, PAK1 | ? |
| Activated transcription factors | NF-κB, CREB, NFAT, SRE | CREB | CREB | AP-1, NF-κB, CREB, NFAT, HIF1α, STAT3, SRE | NF-κB, CREB |
| Modulated target genes | ? | ? | CCL5, FOG2 | Angiogenic factors (VEGF, VEGFR2, TNFα), inflammatory proteins (COX-2, IL-2, IL-4, IL-6, CXCL8, CCL5, GM-CSF), adhesion molecules (ICAM, VCAM), chemokines (CCL2, 3, 4, 7 | PKR, MHC Class I |
| Potential diseases induced by the receptor | Atherosclerosis, oncogenesis | ? | ? | Oncogenesis | ? |

**Table 3.** Signaling abilites of viral chemokine receptors and determined functions in pathological settings. AP-1, activator protein 1; COX-2, cyclooxygenase-2; CREB, cAMP responsive element binding protein; FAK, focal adhesion kinase; FOG, zinc finger protein, multitype 2; GM-CSF, granulocyte-macrophage colony-stimulating-factor; HIF1a, hypoxia inducible factor 1a; ICAM, intercellular adhesion molecule; IL, interleukin; JKN, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T cells; PKR, RNA-dependent protein kinase; SRE, serum responsive element; STAT3, signal transducer and activator of transcription 3; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

Intervention with ORF74 expression or signaling during the early stages of the disease (angioproliferative phase) has been effective (Montaner, Sodhi et al. 2003; Sodhi, Montaner et al. 2004; Jensen, Manfra et al. 2005; Thirunarayanan, Cifire et al. 2007). These studies indicate that ORF74 or ORF74-mediated signaling might be an attractive drug target in the treatment of KS. However, it remains unclear whether intervention during later stages of the diseases, when few cells express ORF74, will be effective.

## Concluding remarks

The different herpesvirus-derived chemokine receptors have been hypothesized to be involved in a plethora of biological processes (Figure 3). One of their main hallmarks is to signal in a ligand-independent, i.e. constitutive manner. As such, they can promote or repress the transcription of various cellular targets. For instance, the HHV-8-encoded ORF74 receptor upregulates various inflammatory and survival factors to promote viral oncogenesis. The constitutive activation of signaling pathways by BILF1 (from HHV-4) or U51 (from HHV-6) may help the virus to evade from the host immune system rather than to promote viral pathologies.

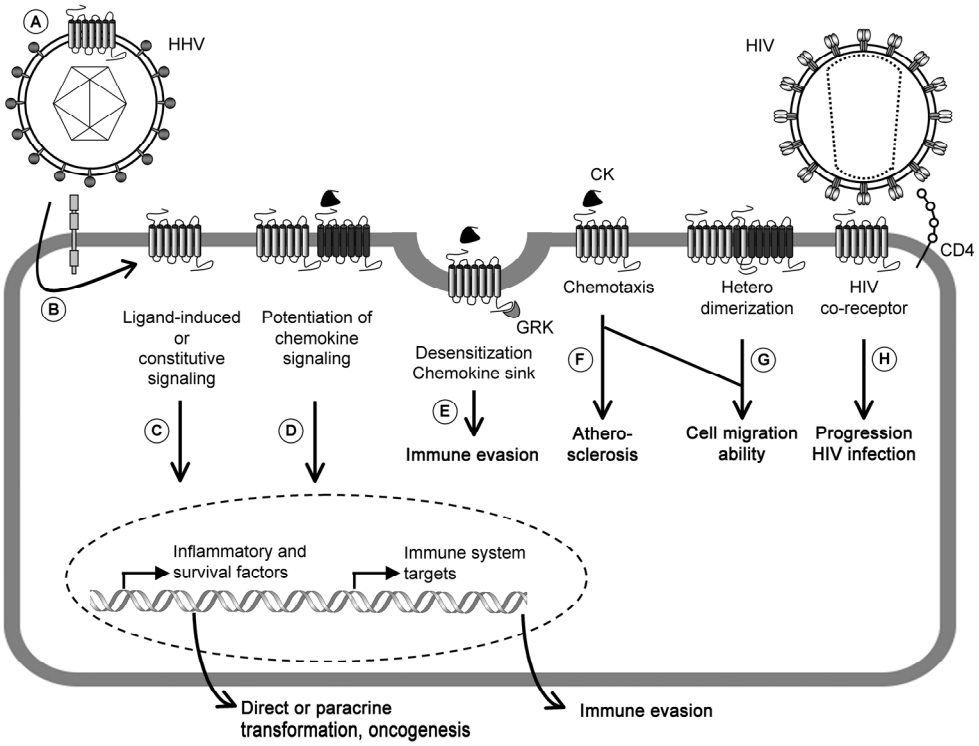


**Figure 3.** Schematic representation of the implication of viral chemokine receptors in various biological events and pathologies. A, viral chemokine receptors are encoded in the viral genome but some of them are present on the virion and may participate in cell adhesion during infection (US28, UL33, US27). B, upon binding to its cellular coreceptor, the virus enters the cell by endocytosis and viral genes, e,g, viral chemokine receptors, are expressed on the surface of the infected cell using the host cellular machinery. C, viral chemokine receptors signal in a ligand-independent or ligand-dependent manner. Constitutive activation of signaling pathways leads to the transcriptional modulation of several target genes, including inflammatory and survival factors, as well as targets of the immune system. Modulation of target genes have implications in transformation and oncogenesis (US28), or in the evasion of the immune system (BILF1, U51). D, viral chemokine receptors also enhance ligand-stimulated signaling of coexpressed endogenous chemokine receptors. This leads to increased activation of transcription factors, e.g. NF-κB (US28). E, endogenous human chemokines are scavenged and can be constitutively internalized by viral chemokine receptors in order to reduce inflammatory signals and escape immune surveillance. F, Chemokine stimulation induces chemotaxis of cells expressing viral GPCRs, altering the normal migration ability of the targeted cell. In a pathophysiological setting, viral chemokine receptors may in turn play a role in the dissemination of the virus (all deorphanized viral chemokine receptors), or in the development of virus-induced cardiovascular conditions, e.g. atherosclerosis (US28, M33, R33). G, viral chemokine receptors were shown to heterodimerize with endogenous chemokine receptors, potentially modifying the natural migration abilities of the cell (BILF1). H, Besides using endogenous chemokine receptors, HIV-1 also utilizes US28 as cellular coreceptor in CD4+ cells. This may in turn facilitate HIV infection.

# II

# Pharmacological and biochemical characterization of human cytomegalovirus-encoded G protein-coupled receptors

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**Abstract**

Human cytomegalovirus (HCMV) is a widely spread herpesvirus that can have serious consequences in immunocompromised hosts. Interestingly, HCMV genome encodes for four viral G protein-coupled receptors (vGPCRs), namely US27, US28, UL33 and UL78. So far, US28 and UL33 have been shown to activate signaling pathways in a ligand-independent manner but US28 remains the best characterized vGPCR and it has been shown to be potentially involved in the development of HCMV-related diseases. As such, detailed investigation of these viral GPCR is of importance in order to understand molecular events occurring during viral pathogenesis and the potential identification of novel therapeutic targets. Herewith we describe several approaches to study these HCMV-encoded vGPCRs. Using molecular biology, tags can be introduced in the vGPCRs which may facilitate the study of their protein expression with various techniques, e.g. microscopy, western-blotting, enzyme-linked immunosorbent assay and flow cytometry. Furthermore, radioligand binding studies can be performed to screen for ligands for vGPCRs but also to study kinetics of internalization. We also describe several signal transduction assays that can evaluate the signaling activity of these vGPCRs. In addition, we discuss oncogenic assays such as foci formation assay and *in vivo* xenograft models that were used to identify US28 oncogenic potential. Finally, the study of these vGPCRs in their viral context can be examined using recombinant HCMV strains generated by bacterial artificial chromosome mutagenesis, and how these mutants can be used in several pharmacological and biochemical assays.

## Introduction

Human cytomegalovirus (HCMV), a member of the human β-herpesvirus family, also referred to as human herpesvirus-5 (HHV-5), is widely spread amongst the population. Although its presence is mostly asymptomatic in immunocompetent hosts, HCMV-positive immunosuppressed patients are at stake with the development of serious inflammatory diseases (Soderberg-Naucler 2006). Futhermore, HCMV has been linked to the development of proliferative diseases, e.g. colon cancer and glioblastoma (Cobbs, Harkins et al. 2002; Harkins, Volk et al. 2002). While the Kaposi's sarcoma-associated herpesvirus (KSHV) and the Epstein-Barr virus (EBV) are considered oncogenic viruses, HCMV appears to preferentially infect cancer cells to further increase their malignant phenotype (Cinatl, Vogel et al. 2004).

Interestingly, like other herpesviruses, the HCMV genome encodes viral G protein-coupled receptors (vGPCRs), referred to as US27, US28, UL33 and UL78, that appear on the surface of human cells upon viral infection (Figure 1A) (Rosenkilde, Smit et al. 2008). So far, UL33 and US28 have been shown to constitutively activate various signaling pathways in a ligand-independent manner (Casarosa, Bakker et al. 2001; Casarosa, Gruijthuijsen et al. 2003). The significance of vGPCR in viral pathogenesis is exemplified by the work on the KSHV-encoded GPCR ORF74. This receptor possesses constitutive activity as well as ligand-induced signaling properties. *In vitro* assays first demonstrated the transforming properties of ORF74 (Bais, Santomasso et al. 1998), and development of transgenic animal models confirmed the ability of ORF74 to induce Kaposi's sarcoma-like diseases (Yang, Chen et al. 2000). As such, the KSHV-encoded vGPCR revealed to be a key player in viral diseases and highlights the importance of vGPCRs in the pathologies of herpesviruses.

Amongst the four vGPCRs encoded by HCMV, US28 is the most extensively studied. US28 binds several chemokines from the CC and CX3C families and was suggested to act as a chemokine sink (Bodaghi, Jones et al. 1998; Kledal, Rosenkilde et al. 1998). In addition US28 constitutively activates the phospholipase C and the NF-κB transcription factor (Figure 1B) (Casarosa, Bakker et al. 2001). Based on these findings, a potential involvement of US28 in HCMV-related diseases has been suggested. For instance, ligand stimulation of US28 showed that it can induce migration of smooth muscle cells, providing a rational for the implication of US28 in the pathogenesis of cardiovascular diseases (Streblow, Soderberg-Naucler et al. 1999). We also demonstrated that the constitutive activity of US28 is responsible for the formation of tumors in a xenograft model, implying that US28 may be an

oncomodulatory viral protein (Maussang, Verzijl et al. 2006). Studies of the other HCMV-encoded GPCRs are still required to elucidate their role during viral infection and their importance in the development of viral diseases.

In this chapter we describe several techniques that can be applied to study virally-encoded GPCRs in more detail. Different research questions can be addressed using molecular, cellular, as well as viral techniques.

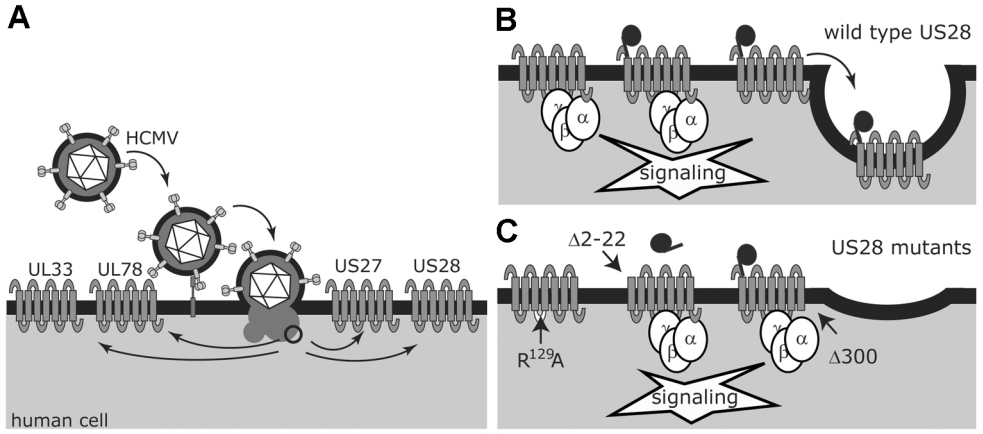


**Figure 1.** Expression and signaling of vGPCRs. **A**, HCMV infection of human cells leads to the expression of the four viral GPCR US27, US28, UL33 and UL78. **B**, US28 signals both in a ligand-independent and dependent manner and undergoes rapid constitutive internalization. **C**, US28 mutants show different signaling and internalization properties than the wild type receptor. US28-$R^{129}A$ does not couple to G proteins and presents no constitutive activity. Δ2-22-US28 still shows constitutive activity but can no longer bind chemokines. US28-Δ300 still binds chemokines and exhibits a higher constitutive activity due to a reduced internalization rate compared to the wild type receptor.

## I. Virally-encoded GPCR engineering

The HCMV-encoded GPCR US28 displays ligand-dependent and independent signaling properties, possesses a broad spectrum of chemokine binding capacity and shows constitutive internalization (Vischer, Leurs et al. 2006). UL33 signals and internalizes in a constitutive manner, while US27 and UL78 appear silent. All three vGPCRs are so far orphans since no ligands have been found to bind these receptors. In order to dissect the contribution of chemokine binding, various US28 mutants were generated (Figure 1C). Truncation

of the N- terminus of US28 by deleting amino acid residues 2-22 (i.e. ∆(2-22)-US28) results in a mutant incapable of binding chemokines but that still presents constitutive activity (Casarosa, Menge et al. 2003; Stropes and Miller 2008). Ala-substitution of the Arg3.50/129 of the conserved DRY motif at the bottom of transmembrane helix 3 (i.e. US28-$R^{129}$A) impairs G protein-mediated signaling without affecting chemokine binding and constitutive internalization (Waldhoer, Casarosa et al. 2003; Stropes and Miller 2008). Constitutive receptor phosphorylation and internalization is attenuated by Ala-substitution of all Ser and Thr residues in the intracellular C-terminal tail or truncation of this domain by deleting the last 54 amino acid residues (i.e. US28-∆300) (Mokros, Rehm et al. 2002; Miller, Houtz et al. 2003; Waldhoer, Casarosa et al. 2003).

Since high quality antibodies against the majority of GPCRs, including the HCMV-encoded receptors, are not available, N-terminal epitope tagged (e.g.) receptors are generated. Tags such as hemaglutinin, FLAG, or c-myc are used to allow detection of expression of vGPCRs with commercially available high affinity antibodies in different assays such as microscopy, Western-blotting, fluorescent-activated cell sorting (FACS) analysis or enzyme-linked immunosorbent assay (ELISA) (McIlhinney 2004). Various epitope tags have been successfully fused to the N-terminus of US28 (Fraile-Ramos, Pelchen-Matthews et al. 2002; Casarosa, Menge et al. 2003; Miller, Houtz et al. 2003; Waldhoer, Casarosa et al. 2003), US27 (Fraile-Ramos, Pelchen-Matthews et al. 2002), and UL33 (Margulies, Browne et al. 1996). Alternatively, engineered variants of green fluorescent protein (GFP) from the jellyfish Aequorea victoria have been genetically fused, in frame by replacing the stopcodon, to the C-terminus of HCMV-encoded GPCRs, allowing localization studies by means of fluorescent (confocal) microscopy (Fraile-Ramos, Kledal et al. 2001; Fraile-Ramos, Pelchen-Matthews et al. 2002; Waldhoer, Kledal et al. 2002; Waldhoer, Casarosa et al. 2003; Stropes and Miller 2008). The most optimal tag needs to be empirically determined for each receptor and should not interfere significantly with ligand binding, receptor signaling and expression.

Various methodologies can be used to introduce site-directed mutations, epitope tags, or to generate fusion proteins (Blomenröhr, Vischer et al. 2004; McIlhinney 2004). Nonetheless, PCR-based approaches using high fidelity DNA polymerase (e.g. Pfu) can be used universally to generate all these different constructs. Short N-terminal tags are introduced after the initial methionine of a vGPCR by using chimeric forward primer (Tf), consisting of the tag-encoding sequence at the 5´-end and a fully complementary GPCR-specific sequence at the 3´-end, in combination

with a reverse open-reading frame (ORF) primer (Or) in a single PCR (Figure 2A).

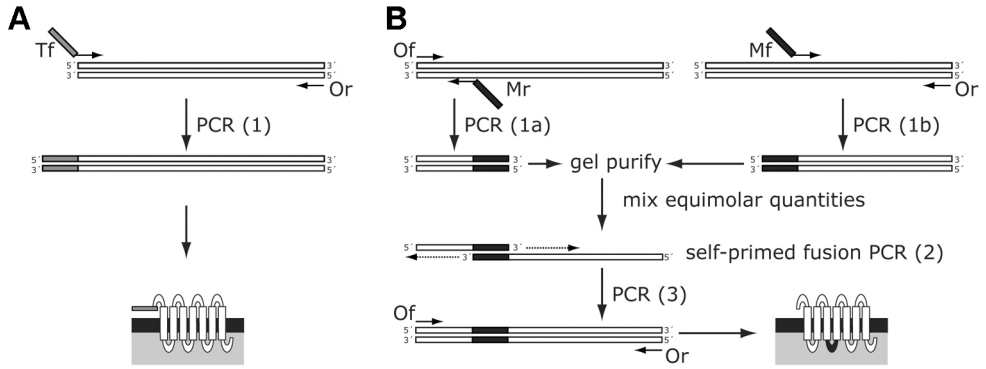


**Figure 2.** Generation of mutated vGPCR using polymerase chain reactions. **A**, N-terminal tagged GPCR are created by PCR using a forward primer (Tf) containing the tag-encoding sequence at the 5´-end and a fully complementary GPCR-specific sequence at the 3´-end, in combination with a reverse open-reading frame (ORF) primer (Or). **B**, 3-step PCR strategy for the creation of a point mutation. In the first PCR, the 5´- and 3´-end cDNA fragments are generated in parallel using overlapping reverse (Mr) and forward (Mf) mutation primers in combination with forward (Of) and reverse (Or) ORF primers, respectively. The two PCR fragments are then fused in a self-primed PCR taking advantage of the introduced overlapping sequences. Next, the fusion products are amplified using the primers Of and Or.

Site-directed mutations are introduced using a 3-step PCR strategy (Figure 2B). In the first PCR, the 5´- and 3´-end cDNA fragments are generated in parallel by using overlapping reverse (Mr) and forward (Mf) mutation primers in combination with forward (Of) and reverse (Or) ORF primers, respectively. The two PCR fragments are then fused in a self-primed PCR taking advantage of the introduced overlapping sequences. Next, the fusion products are amplified in a third PCR using the primers Of and Or (Blomenröhr, Vischer et al. 2004). In principle, this 3-step PCR approach can also be used to generate GPCR-GFP fusion proteins. However, such fusion proteins are more easily generated by substituting the stop codon of the GPCR with a restriction-endonuclease (RE) site, which is also introduced at the 5´-end of the GFP-encoding cDNA. The GPCR-GFP fusion protein is then generated by ligation of both cDNAs.

## II. vGPCR expression, trafficking and radioligand binding

### Microscopic visualization of the cellular localization of vGPCRs

HEK 293T or COS-7 cells that are transiently transfected with vGPCR-GFP fusion protein constructs using 25-kDa linear polyethylenimine (PEI) or DEAE-dextran, respectively (Casarosa, Waldhoer et al. 2005; Verzijl, Storelli et al. 2008), are grown on poly-L-lysine-coated coverslips. Cells are washed with phosphate buffered saline (PBS) and subsequently fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Next, the cells are mounted in Vectashield mounting medium (Vector Laboratories) and analyzed using a confocal laser scanning microscope (e.g. Zeiss LSM 510) with excitation at 505 nm and emission at 530 nm.

### Enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) can be used to monitor membrane and intracellular expression of vGPCRs. HEK 293T or COS-7 cells transfected with epitope-tagged GPCRs are seeded in poly-L-lysine-coated 24-well plates ($2.5 \times 10^5$ and $1.5 \times 10^5$ cells/well, respectively). The next day, cells are fixed using 4% paraformaldehyde in PBS. Samples are then washed with Tris-buffered saline (TBS). Half of the wells can be permeabilized with 0.5% Nonidet P-40 in TBS to detect intracellularly localized GPCRs. After blocking non-specific sites with 1% nonfat-dried milk in 0.1 M $NaHCO_3$, pH 8.6 for 1 h, cells are incubated with the anti-epitope tag antibody in TBS containing 0.1% BSA for 1.5 h at room temperature or overnight at 4°C. Next, the cells are washed three times with TBS, and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody in 1% nonfat-dried milk in 0.1 M $NaHCO_3$, pH 8.6, for 1.5 h at room temperature. Unbound antibodies are washed away with TBS and peroxidase activity is visualized using 3,3´,5,5´-tetramethylbenzidine liquid substrate system (Sigma-Aldrich). Reactions are terminated by adding 0.5 M $H_2SO_4$ and absorption is measured at 450 nm using a Victor$^2$ 1420 multilabel plate reader (Figure 3A).

### Radioligand binding assays

Direct interactions between chemokine ligands and US28 can be quantified using radioligand binding studies. Radiolabeled human chemokines ($^{125}$I) are commercially available from PerkinElmer or can be iodinated in-house using Pierce Iodination Reagent or Bolton-Hunter

Reagents (Daugherty, Siciliano et al. 2000). Three distinct types of radioligand binding experiments can be performed: kinetic, saturation, and competition binding (Bylund, Deupree et al. 2004). Kinetic binding experiments measure the rate of ligand-receptor complex formation and/or dissociation in time. Saturation binding experiments measure the equilibrium binding of increasing concentrations of radioligand and are used to determine the affinity ($K_d$) of the radioligand for a receptor and the number of receptors ($B_{max}$) in a sample. In competition binding experiments, the equilibrium binding of a single concentration radioligand is measured in the presence of increasing concentrations of an unlabeled ligand, allowing determination of the affinity ($K_i$) of numerous unlabeled ligands for a receptor. Radioligand binding assays can be performed on intact cells or membrane preparations. Membrane preparations are commonly used in high-throughput drug screens, whereas the more cumbersome intact cell binding assays allows quantification of receptor cell surface levels and internalization kinetics. Membranes are prepared from e.g. US28-transfected HEK 293T or COS-7 cells. Two days after transfection, the cells are harvested in ice-cold PBS supplemented with 1 mM EDTA, and centrifuged at 1500 g for 10 min at 4°C. Pellets are washed once in the same buffer and subsequently resuspended and homogenized in ice-cold membrane buffer (15 mM Tris, pH 7.5, 1 mM EGTA, 0.3 mM EDTA, and 2 mM $MgCl_2$) using a motorized Teflon-glass homogenizer (10 strokes at ~1200 rpm). Membranes are then subjected to two freeze-thaw cycles using liquid nitrogen and subsequently centrifuged at 40,000 g for 25 min at 4°C. Pellets are washed once with ice-cold Tris-sucrose buffer (20 mM Tris, pH 7.4, and 250 mM sucrose), before being resuspended in the same buffer and frozen in liquid nitrogen. For competition binding experiments in 96-well microplates, 25 µl $^{125}$I-chemokine (~0.25 nM/well) in binding buffer (50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 100 mM NaCl, and 0.5% BSA) is dispensed together with 25 µl of increasing concentrations unlabeled ligand in each well. Next, binding reactions are initiated by adding 50 µl of purified membrane (~0.5–10 µg membrane protein/well) and incubated for 2 hr at room temperature with gentle agitation. The optimal amount of membrane protein and concentration of $^{125}$I-chemokine (~0.3-0.7 x $K_d$) need to be empirically determined in order to obtain a maximal detection window without binding more than 10% of the radioligand. Incubations are terminated by filtration through a UniFilter-96 GF/C (Perkin-Elmer) presoaked in 0.3% PEI, and subsequently washed with ice-cold binding buffer supplemented with 0.5 M NaCl using a Filtermate Harvester (Perkin-Elmer). Radioactivity is quantified by liquid scintillation using a Wallac MicroBeta TriLux (Perkin-Elmer). Next, radioligand binding is plotted as function of the logarithm of the unlabeled ligand concentration (Figure

3B), and $IC_{50}$ values are determined by non-linear curve fitting using GraphPad Prism. The affinity of the unlabeled ligand ($K_i$) is calculated using the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + [^{125}I\text{-chemokine}]/K_d)$ (Cheng and Prusoff 1973).

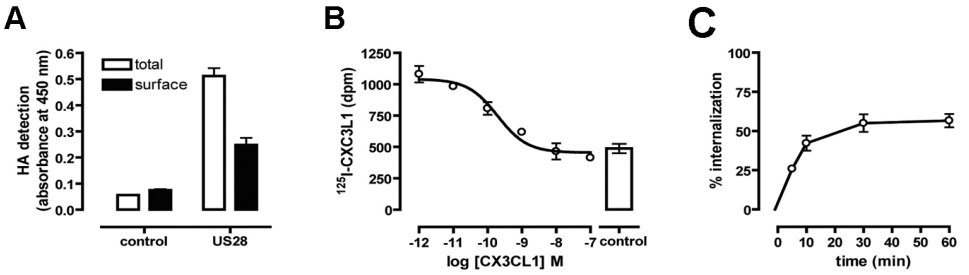


**Figure 3.** US28 protein expression and internalization in transfected cells. **A**, Hemagglutinin-tagged US28 (HA-US28) encoding plasmid is transfected into HEK 293T cells. 24h later, the epitope-tagged protein is detected using an ELISA assay against the HA tag. **B**, radiolabeled [$^{125}$I]-CX3CL1 binds to US28-expressing membranes. Cold CX3CL1 displaces the radioligand in a dose-dependent manner down to the level observed in membranes prepared from mock-transfected control cells. **C**, internalization studies indicate that US28 rapidly internalizes radiolabeled chemokines as soon as 5 min after their addition at 37°C.

## Internalization assays

US28 acts as a decoy receptor for many inflammatory chemokines by removing them from the microenvironment of HCMV-infected cells through rapid and constitutive internalization. As such, US28 may attenuate the inflammatory response by reducing the recruitment of chemokine-responding inflammatory cells (Bodaghi, Jones et al. 1998; Billstrom, Lehman et al. 1999; Fraile-Ramos, Kledal et al. 2001; Randolph-Habecker, Rahill et al. 2002). Internalization kinetics can be monitored by quantification of $^{125}$I-chemokine uptake by US28-expressing cells. To this end, transiently US28-expressing HEK 293T ($1{,}6 \times 10^5$ cells/well) are seeded in poly-L-lysine-coated 48-well plates. The next day, medium is aspirated and cells are incubated at 37°C with 0.25 nM $^{125}$I-chemokine in pre-warmed binding buffer using time intervals ranging from 5 min to 1 h. Incubations are terminated by placing the plates on ice and immediately washing the cells three times with ice-cold binding buffer supplemented with 0.5 M NaCl. For each time point, total radioactivity was determined by collecting one set of cells in lysis buffer (0.5% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholic acid), whereas a second set of cells was first incubated for 10 min in ice-cold acidified DMEM (pH 2.0) to remove

surface-bound chemokine before being collected in lysis buffer. Control experiments revealed that the acidic incubation removed all surface-bound chemokine while leaving the receptor surface intact. Next, radioactivity in collected cell lysates is quantified using a Wallac Compugamma counter (PerkinElmer). The percentage of $^{125}$I-chemokine internalization is calculated for each time point using: internalization (%) = (acid-resistant radioactivity/total radioactivity) x 100 (Figure 3C).

## III. vGPCR-induced signal transduction

### Inositol phosphate production

Both UL33 and US28 constitutively activate the enzyme phospholipase Cβ to produce inositol triphosphate ($InsP_3$) and diacylglycerol by hydrolyzing plasma membrane phosphatidylinositol 4,5-bisphosphates ($PIP_2$). Prelabeling the cells overnight with myo-[2-$^3$H]-inositol allows metabolic incorporation into $PIP_2$. PLCβ-catalyzed production of $^3$H-$InsP_3$ is measured in the presence of lithium, which inhibits the rapid dephosphorylation of InsP to inositol, resulting in the accumulation of $^3$H-InsP (Huckle and Conn 1987). HEK 293T (~5 x $10^4$ cells/well) or COS-7 cells (~3 x $10^4$ cells/well) that are transiently transfected with US28 or UL33, or SVEC4-10 cells (~5 x $10^4$ cells/well) stably expressing US28 are seeded in poly-L-lysine-coated 96-well plates and incubated overnight in 100 µl/well Earle´s inositol-free minimal essential medium (Invitrogen) supplemented with 10 µCi/ml myo-[2-$^3$H]-inositol (17 Ci/mmol; GE Healthcare). Importantly, overnight labeling of HEK 293T cells requires the supplementation of medium with 10% fetal bovine serum. The next day, cells are washed with DMEM supplemented with 25 mM Hepes (pH 7.4) and 20 mM LiCl, and subsequently incubated in the same medium in the absence or presence of ligands at 37°C for 2 h. Incubations are terminated by aspiration of the medium and cellular lipids are extracted from the cells using 10 mM formic acid. [$^3$H]-InsP accumulation is then quantified using 0.5 mg/well YSi-RNA-binding SPA beads (GE Healthcare) in white clear-bottomed 96-well isoplates using a Wallac MicroBeta Trilux counter (PerkinElmer) (Figure 4A) (Brandish, Hill et al. 2003). This assay can be used for US28 to screen for inverse agonist properties of chemokine ligands, e.g. CX3CL1 or small compounds (Hulshof, Vischer et al. 2006).

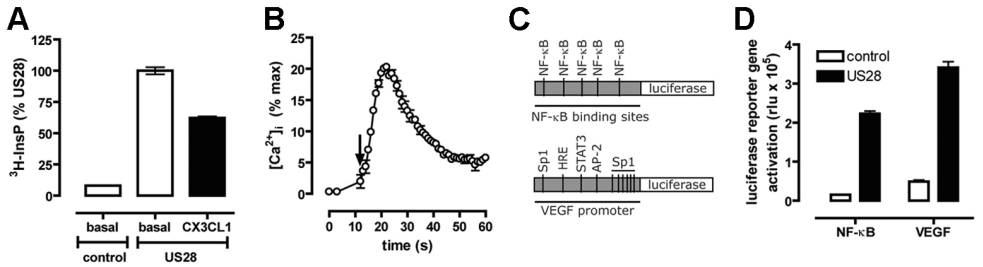


**Figure 4.** US28 signals in both a ligand-dependent and independent manner. **A**, US28-expressing SVEC 4-10 cells present a ligand-independent formation of inositol phosphate (InsP) compared to mock-transfected cells. Incubation of US28-expressing cells with CX3CL1 can partially inhibit this constitutive signaling. **B**, stimulation of SVEC 4-10 cells stably expressing US28 with CCL5 induces a transient increase in intracellular calcium signaling ($[Ca^{2+}]_i$). **C**, schematic representation of the different transcription factors bindings sites controlling the luciferase gene in the NF-κB and the human vascular endothelial growth factor (VEGF) promoter reporter gene plasmids. **D**, HEK 293T cells are transfected with the polyethylenimine (PEI) method with pcDEF3 plasmid either empty (control) or containing the sequence of US28 together with the reporter gene plasmid. The total amount of DNA was kept constant at 2 μg per $10^6$ cells. Plasmid DNA (1 μg reporter gene with 900 ng pcDEF3 and 100 ng pcDEF3 either with or without US28 sequence) is diluted in 75 μl of 150mM NaCl solution and mixed with 75 μl 150 mM NaCl containing 6 μg PEI. HEK 293T cells are harvested and resuspended in culture medium to a concentration of 0,5 x $10^6$ cells per ml. 2 ml cell suspension are added to the DNA:PEI mixture and 100 μl of transfected cells are seeded per well of a white 96-well plate. Luminescence is measured 24h later after transfection.

## Intracellular $[Ca^{2+}]$ measurements

US28 induces a rapid transient increase in intracellular $Ca^{2+}$ levels in response to CC and CX3C chemokines (Gao and Murphy 1994; Billstrom, Johnson et al. 1998; Casarosa, Waldhoer et al. 2005). SVEC4-10 cells stably expressing US28 are seeded in clear-bottomed black 96-well plates (4 x $10^4$ cells/well) (Casarosa, Waldhoer et al. 2005). The next day, cells are loaded with 4 μM cell-permeant Fluo-4 acetoxymethyl ester (Invitrogen) in loading buffer (Hanks´ balanced salt solution supplemented with 20 mM Hepes, pH 7.4, 2.5 mM probenecid) supplemented with 0.04% pluronic acid and 1% BSA, for 30 min at 37°C in the dark. Cells are washed twice and preincubated for 1 h at 37°C in the dark in loading buffer supplemented with 0.1% BSA. Intracellular $Ca^{2+}$ levels are monitored at 37°C by measuring fluorescence (excitation at 485 nm and emission at 520 nm) with a Novostar microplate reader (BMG Labtechnologies GmBH, Offenburg) for 10 s to determine mean basal level. Next, the chemokine is injected and fluorescence is recorded for another 50 s, after which cells are lysed by adding 5% Triton X-100

to determine maximum fluorescence. Results are expressed as percentage of maximum fluorescence (Figure 4B).

**Reporter gene assays**

Reporter gene assays are commonly used to determine the signaling properties and functional effects of GPCRs. Whether their viral counterparts are constitutively active or can be stimulated with ligands (when known), the transcriptional activity or direct transcription of various cellular factors can be analyzed. Initial characterization of US28 and UL33 constitutive signaling properties was performed using NF-κB reporter gene assays (Casarosa, Bakker et al. 2001; Casarosa, Gruijthuijsen et al. 2003). This reporter gene plasmid encodes the luciferase gene controlled by five successive NF-κB binding sites (Figure 4C). As such, upon activation of the NF-κB transcription factor, the luciferase gene is transcribed and expressed at the protein level. Alternatively the activation of downstream target genes such as the vascular endothelial growth factor (VEGF) can also be quantified. In that case, the luciferase gene is controlled by the endogenous promoter of the VEGF gene that contains binding sites of various transcription factors (Figure 4C). This method was used to assess the pro-angiogenic properties of US28 (Maussang, Verzijl et al. 2006).

HEK 293T cells are transfected with control or US28 plasmids and with either the NF-κB reporter gene or the human VEGF promoter reporter gene using the PEI method. 24h after transfection, cells are lysed and stimulated with Luciferin (0.83 mM ATP, 0.83 mM D-Luciferin, 18.7 mM $MgCl_2$, 0.78 μM $Na_2H_2P_2O_7$, 38.9 mM Tris (pH 7.8), 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100 and 2.6 μM DTT) and light emission is quantified with a Victor$^2$ (Figure 4D). This method can be extended to other transcription factors such as e.g. cyclic AMP responsive element binding protein (CREB) and nuclear factor of activated T cells (NFAT) (McLean, Holst et al. 2004), and alternatively, the luciferase gene can be replaced by the β-galactosidase reporter gene (Lim, Smits et al. 2006).

## IV. vGPCR-induced oncogenesis

### Cellular transformation: foci formation assay

Cellular transformation induced by human or viral oncogenes is typically assessed using stably transfected NIH-3T3 cells. These mouse fibroblasts are on the verge of transformation and allow sensitive detection of oncogenic signals, resulting in cellular transformation. However, in order to ascertain the oncogenic properties of the studied proteins, mock-transfected cells always have to be taken as a negative control in the experiment to estimate the background activity. NIH-3T3 cells are transfected with a US28-encoding plasmid using the calcium phosphate method (Chen and Okayama 1988). This plasmid also contains the antibiotic resistant gene neomycin allowing the selection of geneticin-resistant US28-expressing cells. NIH-3T3 cells possess cell contact inhibition properties that disable them to proliferate when entering in contact with adjacent cells within a cell monolayer. Upon transformation, cells lose this ability and uncontrolled growth of cells leads to formation of cell foci (Maussang, Verzijl et al. 2006). The transforming ability of US28-expressing NIH-3T3 cells is measured when cells are cultured together with native NIH-3T3 cells that still possess cell contact inhibition. The latter grow in a monolayer, while US28-transformed cells grow on top of one another, leading to the formation of foci. To this end, 2 x $10^5$ naive NIH-3T3 cells are cultured together with 2 x $10^3$ mock or US28 stably transfected NIH-3T3 cells for 14 days in the absence of antibiotic selection. Medium is refreshed biweekly. To detect the formed foci, wash cells twice with PBS, twice with ice-cold methanol and fix them with ice-cold methanol for 5 min. After washing the dish with distilled water, stain the cells with 0.4 % methylene blue for a few minutes. Wash the dishes extensively with distilled water until the rinsing water does not appear blue. Foci are then counted in each sector (Figure 5A).

### Cell proliferation assay: cyclin D1 expression

US28-mediated signaling pathways upregulate the expression of Cyclin D1 (Figure 5B) that is involved in cell cycle progression and proliferation (Maussang, Verzijl et al. 2006). Control or US28 stably transfected NIH-3T3 cells are seeded in a 6-well plate (3 x $10^5$ cells per well) and cultured overnight in DMEM supplemented with 10 % calf serum. The following day, cells are synchronized in the $G_0$ phase by serum starvation (DMEM + 0.5 % calf serum) overnight. The next day, samples are washed twice with cold PBS and lysed for 10 min on ice with 75 µl

RIPA lysis buffer supplemented with proteases inhibitors. Cell lysates are collected in 1.5 ml tubes, sonicated for 3 sec and subsequently centrifuged at 15 000 g for 10 min at 4°C. Next, collect supernatant and use an aliquot to determine protein concentration using commercially available kits and store the remaining protein sample at -80°C. Load equal amounts of proteins onto a 10% SDS-PAGE electrophoresis gel and run at constant voltage (100V) for approximately 1.5 h. Transfer the proteins from the electrophoresis gel onto a PVDF membrane for 1h at constant intensity (200 mA) and block the membrane in blocking buffer containing 5% dry milk for at least 1 h. Determine the Cyclin D1 protein levels using a mouse anti-Cyclin D1 (Upstate Millipor, Cat#05-815) as primary antibody (at 4°C overnight), and an HRP-conjugated goat anti-mouse antibody (BioRad, Cat#170-6516) as secondary antibody (at room temperature for 1 h). HRP-derived chemiluminescence is measured using standard commercially available kits (e.g. ECL kit, Amersham) and Imaging films (e.g. Kodak BioMax Light films). To verify that the levels of proteins are equal in all lanes, the blot is stripped using 0.2 N NaOH solution, blocked with 5% dry milk in blocking buffer, and probed for β-actin levels (Sigma, Cat#A5440) (Smit, Bakker et al. 2002).

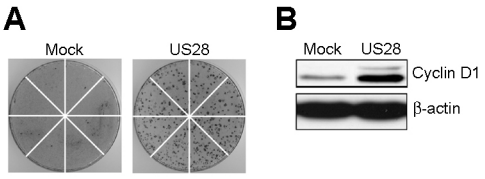


**Figure 5.** US28 induces a transformed phenotype and increased proliferation in NIH-3T3 cells. **A**, 2 x $10^3$ NIH-3T3 cells stably transfected with either mock (empty plasmid) or US28 are grown during 2 weeks together with 2 x $10^5$ naive NIH-3T3 cells. The formed foci are stained with methylene blue. **B**, Total lysates from mock and US28-stably transfected NIH-3T3 cells present higher expression levels of Cyclin D1. Protein levels are normalized against β-actin expression.

### *In vivo* xenograft models

The tumorigenic character of vGPCRs can be confirmed using tumor xenograft models in nude mice. These mice are deprived in T cells and are consequently not able to mount T cell-mediated immune responses, such as graft rejection. To this end, 2 x $10^6$ NIH-3T3 cells stably transfected with either mock or US28 are injected subcutaneously in each flank of the animal and tumor formation is checked every other day. Each injected side is considered as an independent tumor. The length, width and depth of growing malignancies are measured with a caliper and the volume of the tumors is determined by calculating the half of the product of the three dimensions of the tumor. Malignancies

can be considered as tumors when their sizes are superior or equal to 50 $mm^3$. Tumor growth can be depicted as the tumor size upon time post-injection (Figure 6A), or by means of Kaplan-Meier curves to illustrate the percentage of mice presenting tumors over time (Figure 6B).

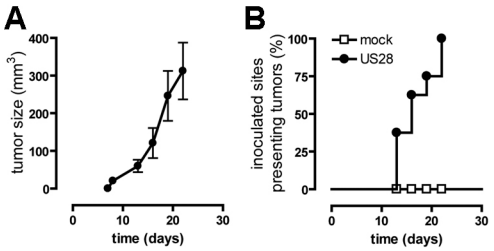


**Figure 6.** Representation of US28-induced tumor formation in xenograft models. **A**, tumors formed in the flanks of nude mice injected with 2 x $10^6$ US28-stably transfected NIH-3T3 cells are measured with a caliper and the tumor volume is calculated as the half product of the length, width and depth. Tumor formation is followed over time after the injection of stably transfected cells. **B**, Kaplan-Meier curves are used to determine the percentage of animals presenting tumors larger than 50 $mm^3$ and show that US28-induced tumors have a 100% incidence within 21 days post-injection.

## V. Generation of recombinant HCMV strains by markerless bacterial artificial chromosome mutagenesis

Bacterial artificial chromosome (BAC) mutagenesis has become an excellent tool to manipulate the CMV genome and to investigate the function of vGPCRs in the context of viral infection in biologically relevant cells (specifically endothelial cells and macrophages). The generation of recombinant CMVs by BAC mutagenesis has been achieved by several research groups investigating vGPCRs (e.g. MCMV M33 (Davis-Poynter, Lynch et al. 1997), RCMV R33 (Beisser, Vink et al. 1998), HCMV US28 (Minisini, Tulone et al. 2003), HCMV UL33 (Casarosa, Gruijthuijsen et al. 2003), RCMV R78 (Kaptein, Beisser et al. 2003) and HCMV UL78 (Michel, Milotic et al. 2005)). In particular, the publication of Streblow et al. in 1999 highlighted the importance for vGPCR research by demonstrating that US28 is responsible for the migration of HCMV-infected smooth muscle cells (Streblow, Soderberg-Naucler et al. 1999).

The ground for recombinant CMVs was prepared by cloning of CMV genomes from various species into BACs (Messerle, Crnkovic et al. 1997; Brune, Messerle et al. 2000). Initally, BAC mutagenesis was achieved by means of shuttle plasmids using RecA-mediated recombination with homologous flanks of 500 to 3000 bp (Casarosa,

Gruijthuijsen et al. 2003; Michel, Milotic et al. 2005), but this method was very time-consuming. Later, the establishment of the Red-mediated or RecE/T-recombination to manipulate BACs provided a reliable faster technique (Borst, Mathys et al. 2001; Wagner, Ruzsics et al. 2002). However, since this method led to the persistence of undesired genetic sequences, it was not an ideal tool to generate point mutations or introduce molecular tags to the target gene. This technique has further been optimized (Warming, Costantino et al. 2005; Tischer, von Einem et al. 2006) and the so-called "en passant" mutagenesis described by Tischer et al. in 2006 is a powerful tool for the traceless introduction of potentially any mutation into the CMV genome. Markerless BAC mutagenesis is based on two recombination steps: (i) the insertion of the mutation at the target site and (ii) the excision of the positive (kanamycin resistance) and the negative (I-SceI) selection marker. Briefly, a linear DNA fragment containing the sequences needed for the two recombination steps (the directed integration and precise excision of the unwanted sequences) is generated by PCR (Tischer, von Einem et al. 2006). It is then electroporated into recombination competent E.coli, harbouring (i) a CMV BAC genome, (ii) the Red recombination system under control of a temperature sensitive promoter and (iii) the coding sequence for the homing endonuclease I-SceI under control of an arabinose inducible promoter. Successful integrates, controlled by PCR and restriction fragment length polymorphism (RFLP) analysis, undergo the second round of recombination removing the negative and positive selection marker. This step is performed by induction of the Red recombination system at 42°C for 20 min and the parallel induction of I-SceI endonuclease by 1% arabinose, resulting in a mutated BAC carrying only the CMV genome with the desired mutation. The mutated BAC is checked again by PCR, RFLP with at least three restriction enzymes and sequencing of the mutated region. Recombinant virus is reconstituted by electroporation of the mutated BAC DNA into CMV permissive cells (Sinzger, Hahn et al. 2008; Chevillotte, Landwehr et al. 2009). "En passant" mutagenesis offers two major advantages: (i) recombinant BACs can be generated within less than 14 days and (ii) the method can be applied sequentially for the generation of mutations in any order.

Using BAC mutagenesis, we generated different mutants derived from AD169 and TB40 HCMV strains. AD169 strains lacking US28 or also four vGPCRs (US27, US28, UL33 and UL78) demonstrate that US28 is responsible for the observed CCL5 binding (Figure 7A) and constitutive inositol phosphate formation in infected human foreskin fibroblasts (HFF) (Figure 7B). Furthermore, infection of human glioblastoma U373 cells with the Titan strain induces the activation of the human VEGF

promoter. After deletion of US28, the observed pro-angiogenic phenotype is impaired, highlighting the potential involvement of US28 in HCMV-related pathogenic conditions (Figure 7C).

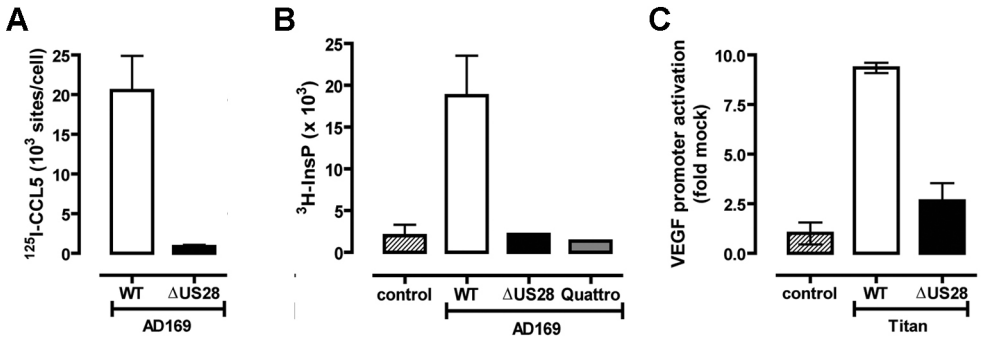


**Figure 7.** Cell surface expression and signaling properties of vGPCRs in HCMV-infected cells. **A**, human foreskin fibroblasts (HFF) are infected with the AD169 WT and ΔUS28 (lacking the US28 gene) strains. 8 h post-infection, [$^{125}$I]-CCL5 specific binding is detected in cells infected with the WT virus, but it is almost completely abrogated in cells infected with the ΔUS28 mutant. **B**, infection of HFF cells with HCMV strain AD169 leads to a constitutive formation of inositol phosphate (InsP) 48 h post infection. Deletion of either US28 only (ΔUS28) or the 4 vGPCRs US27, US28, UL33 and UL78 (Quattro) completely impairs InsP accumulation (Jens Holl, Andreas Schreiber and Detlef Michel, unpublished data). **C**, human glioblastoma U373 cells infected with the HCMV Titan strain present an increased activation of the human VEGF promoter which is impaired after deletion of US28 gene.

## Concluding remarks

The study of HCMV-encoded vGPCRs can be performed at several levels. For the quest of nonpeptidergic drug-like compounds that can inhibit US28-mediated activities, high throughput screening methods for InsP formation and radioligand binding have been successfully used. Similar approaches can be used to identify cognate ligands for US27, UL33 and UL78. Signaling assays determine whether these vGPCRs are constitutively active and whether they present ligand-induced signaling properties once these receptors are deorphanized. The BAC mutagenesis method is also a very useful tool to determine the importance of vGPCRs in the context of HCMV-infected cells.

# III

# Human cytomegalovirus-encoded chemokine receptor US28 promotes tumorigenesis

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**Abstract**

Human cytomegalovirus (HCMV) is a widely spread herpesvirus, suggested to play a role in tumor progression. US28, a chemokine receptor encoded by HCMV, binds a broad spectrum of chemokines and constitutively activates various pathways linked to proliferation. Our studies reveal that expression of US28 induces a pro-angiogenic and transformed phenotype by up-regulating the expression of vascular endothelial growth factor and enhancing cell growth and cell cycle progression. US28 expressing cells promote tumorigenesis when injected into nude mice. The G protein-uncoupled constitutively inactive mutant of US28, induces delayed and attenuated tumor formation, indicating the importance of constitutive receptor activity in the early onset of tumor development. Importantly, also in glioblastoma cells infected with the newly isolated clinical HCMV strain Titan, US28 was shown to be involved in the HCMV-induced angiogenic phenotype. Hence, the constitutively activated chemokine receptor US28 might act as a viral oncogene and enhance and/or promote HCMV-associated tumor progression.

## Introduction

Human cytomegalovirus (HCMV) is a widely spread beta-herpesvirus that prevails in 30 up to 90% of the population (Gandhi and Khanna 2004). In immunocompetent hosts, the virus remains in a latent form, but in immunocompromised hosts like organ transplant recipients and individuals with AIDS, HCMV infection can lead to severe pathologies such as pneumonitis, hepatitis and retinitis (Gandhi and Khanna 2004). Moreover, HCMV has been associated with chronic diseases, including e.g. vascular diseases (Stassen, Vega-Cordova et al. 2006) and malignancies, among others colon cancer (Harkins, Volk et al. 2002) and malignant glioma (Cobbs, Harkins et al. 2002). Although the causative role for HCMV in the development of malignancies remains 7to be established, various HCMV proteins and DNA have been detected with high frequency in tumor tissues (Cobbs, Harkins et al. 2002; Harkins, Volk et al. 2002). In addition, it has been shown that HCMV preferentially infects tumor cells as they present a favorable environment for the virus to exert its oncogenic potential (Cinatl, Vogel et al. 2004). HCMV infection upregulates different growth factors and cytokines resulting in enhanced cell survival, proliferation and angiogenesis (Cinatl, Vogel et al. 2004). As such, HCMV appears to enhance the malignant behavior of tumor cells, implying an oncomodulatory role for the virus.

HCMV encodes four G protein-coupled receptors (GPCR), US27, US28, UL33 and UL78, showing highest homology to human chemokine receptors (Chee, Satchwell et al. 1990). This latter class of receptors plays a fundamental role in the control and regulation of the immune system, but some (e.g. CXCR4) have recently been shown to play a prominent role in cancer and more specifically metastasis (Balkwill 2004). In fact, the Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded chemokine receptor ORF74 induces angioproliferative lesions that morphologically resemble Kaposi’s sarcoma-like lesions when expressed *in vivo* (Yang, Chen et al. 2000). As such, ORF74 is regarded as a causative agent of Kaposi’s sarcoma.

The viral chemokine receptor US28 has been by far the most extensively studied of the four HCMV-encoded GPCRs (Vischer, Leurs et al. 2006). It binds a broad spectrum of chemokines including CCL2/MCP-1, CCL5/RANTES and CX3CL1/Fraktalkine, and unlike its cellular homologue CCR1, exhibits constitutive activity (Casarosa, Bakker et al. 2001). US28 has been shown to constitutively activate signaling pathways linked to proliferation and migration when expressed *in vitro*, but also after HCMV infection (Streblow, Soderberg-Naucler et al. 1999; Casarosa, Menge et al. 2003). Moreover, US28 shows promiscuous G

protein coupling, constitutively signaling e.g. through $G\alpha_q$ proteins and is able to potentiate signaling of cellular $G\alpha_i$-linked chemokine receptors (Casarosa, Bakker et al. 2001; Minisini, Tulone et al. 2003; Bakker, Casarosa et al. 2004). Hence, HCMV may effectively use US28 to orchestrate multiple signaling networks within infected cells. US28 might be of key importance in subverting cellular signaling and contribute to the onset and/or progression of tumorigenesis.

To assess the oncogenic potential of US28, we performed *in vitro* and *in vivo* experiments with wild type and constitutively inactive US28 expressing cells. In addition, we examined the angiogenic status of cells, infected with either the newly isolated clinical HCMV strain Titan or its US28 deletion mutant.

## Results

### US28 induces a transformed phenotype in NIH-3T3 cells

To study the role of US28 in cellular transformation, we stably transfected the mouse fibroblast NIH-3T3 cell line with either US28 wild type (WT) or the G protein-uncoupled mutant of US28 (US28-$R^{129}$A), which has previously been shown to be devoid of G protein-mediated signaling (Waldhoer, Casarosa et al. 2003). Different clones of each cell line were generated and various clones were analyzed (all data shown are representative for different clonal cell lines). Clonal cell lines expressing US28-WT or US28-$R^{129}$A, showed specific binding of $^{125}$I-CCL5, while constitutive inositol phosphate (InsP) production was only observed for US28-WT, demonstrating proper surface expression and expected signaling properties of both receptors (Figure 1A). The observed increases in CCL5 binding and inositol phosphate production were comparable to previously published data on HCMV-infected cells (Minisini, Tulone et al. 2003), indicating that the expression levels of US28 in NIH-3T3 cells reflected conditions under viral infection. When cells were cultured in regular medium, the US28-WT expressing cells displayed increased growth rate (Figure 1B). Whereas mock transfected and US28-$R^{129}$A expressing cells stopped growing upon reaching 100% confluency, the growth rate of US28-WT expressing cells was not decreased. These studies were further corroborated by measuring $^{3}$H-thymidine incorporation (Figure 1C). DNA synthesis upon serum starvation (0.5% serum containing medium) was four-fold higher in US28-WT expressing cells than in mock transfected and US28-$R^{129}$A expressing cells. To confirm the oncogenic potential of US28, a focus formation assay was performed, which showed that only US28-WT expressing cells induced foci formation (Figure 1D). The cell lines expressing the G protein-uncoupled mutant US28-$R^{129}$A or mock transfected cell lines showed no foci formation, due to the contact inhibition when cells reached confluency. Additionally, we investigated whether US28 also induces an angiogenic phenotype in the stably transfected NIH-3T3 cells by measuring the production of the angiogenic vascular endothelial growth factor. After 5 days of culture, the US28-WT expressing cells secreted five-fold more VEGF protein compared to mock transfected and US28-$R^{129}$A expressing cells (respective VEGF concentrations were 521 $\pm$ 87 for mock, 2548 $\pm$ 47 for US28-WT cells and 223 $\pm$ 61 pg/ml for US28-$R^{129}$A cells) (Figure 1E). Thus, the constitutive activation of G proteins by US28 promotes a transformed phenotype in the NIH-3T3 cell line, through increased growth rate,

enhanced DNA synthesis and loss of contact inhibition, as well as an pro-angiogenic phenotype mediated by VEGF.

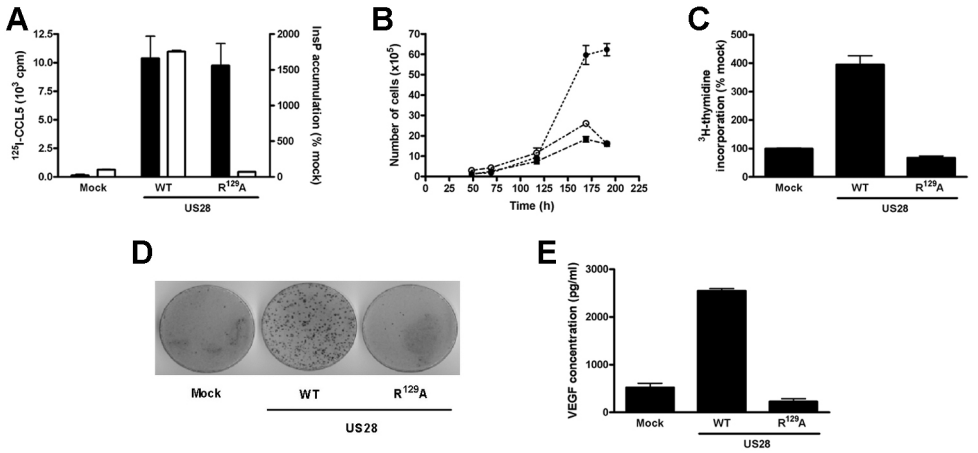


**Figure 1.** US28 induces a transformed and pro-angiogenic phenotype in stably transfected NIH-3T3 cells. **A**, NIH-3T3 cells were stably transfected with either mock, US28-WT or US28-$R^{129}$A (G protein-uncoupled mutant). Both US28 receptors showed specific $^{125}$I-CCL5 binding (black bars), determined with Fraktalkine/CX3CL1 $10^{-7}$M, while only the WT receptor constitutively increased the formation of inositol phosphate (InsP). **B**, representative cell growth curves of mock transfected (■), US28-WT (●) and US28-$R^{129}$A (○) expressing cell lines, showing the enhanced growth of US28-WT expressing cells. **C**, upon serum starvation, the incorporation of $^{3}$H-thymidine was higher in US28-WT expressing cells compared to mock transfected and US28-$R^{129}$A expressing cells. **D**, focus formation assay showing that only US28-WT expressing cells lost their contact inhibition abilities leading to the formation of numerous foci, whereas the mock and inactive mutant (US28-$R^{129}$A) transfected cells only formed a limited number of foci. **E**, When cultured for 5 days, NIH-3T3 cells expressing US28-WT produced higher amounts of vascular endothelial growth factor (VEGF) protein compared to mock transfected and inactive mutant expressing cells. Data represent mean ± s.e.m. of representative experiments.

## US28 enhances the cell cycle progression of NIH-3T3 cells

One of the characteristics of transformed cells is enhanced cell cycle progression. Stably transfected NIH-3T3 cells were assayed to determine the populations of cells present in the different phases of the cell cycle and the proliferation index (P.I.), defined as the ratio between S, $G_2$/M cells and $G_0/G_1$ cells (P.I.=S$xG_2M/G_0G_1$), was calculated. Cells were first synchronized during 24h by serum starvation (0.5% serum containing medium) and further stimulated for 24h using 10% serum containing medium. Cell cycle analysis revealed that the US28-WT expressing cells were more represented in the S and G2/M phases, whereas the G1 population was reduced (Figure 2A showing a representative experiment). As a result, the P.I. was on average twice

as high for US28-WT expressing cells when compared to mock transfected cells (respectively 0.70 ± 0.06 and 0.37 ± 0.03). US28-$R^{129}$A expressing cells had a lower P.I. (0.50 ± 0.06) compared to US28-WT expressing cells. The different P.I. of the three cell lines were significantly different from each other ($p<0.001$) indicating that the US28 proliferative phenotype is not solely G protein-mediated.

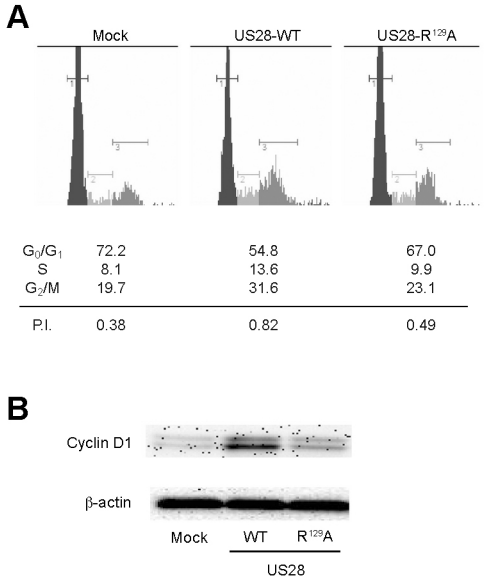


**Figure 2.** US28 enhances proliferation through increased cell cycle progression and Cyclin D1 expression. **A**, stably transfected cells were analyzed with the GUAVA Cell Cycle flowcytometer. After synchronization of the cells by serum starvation, cells were grown in regular medium and US28-WT expressing cells showed an enhanced cell cycle progression compared to mock. US28-$R^{129}$A expressing cells had an intermediate proliferation index in comparison with US28-WT expressing and mock transfected cells. **B**, western blot analysis of total cell lysates showed that US28-WT induced the upregulation of Cyclin D1 compared to mock transfected and US28-$R^{129}$A expressing cells. Data are mean ± s.e.m. of representative experiments.

Increased oncogene-driven cell growth has been attributed to the upregulation of proteins involved in the different check points of the cell cycle, such as Cyclin D1 which mediates the transition from the G1 phase to the S phase (Sherr 1996). Stably transfected NIH-3T3 cells were synchronized in 0.5% serum containing medium for 24h, further stimulated for 24h with regular culture medium and total lysates were analyzed by Western blot for Cyclin D1 expression (Figure 2B showing a representative figure). US28-WT expressing cells clearly showed an upregulation of Cyclin D1 protein expression when compared to mock transfected and US28-$R^{129}$A expressing cells (relative band intensity mock:100 ± 0%, US28-WT: 250 ± 41%, US28-$R^{129}$A: 165 ± 11%).

Thus, the constitutive activity and G protein coupling of US28 appear essential for its transforming potential as measured by the enhanced cell cycle progression and upregulation of Cyclin D1 expression.

**US28 constitutive activity induces VEGF gene expression**

As VEGF protein expression was shown to be constitutively upregulated by US28, we investigated the mechanism by which US28 activated the VEGF promoter. For this purpose, COS-7 cells were transiently transfected with increasing concentrations of $pcDEF_3$ vector containing the hemagglutinin (HA)-tagged wild type (WT) US28 receptor (HA-US28-WT) cDNA. This resulted in a dose-dependent enhancement of cell surface receptor expression, as detected by $^{125}$I-CCL5 binding, as well as in a constitutive increase in InsP formation (data not shown). COS-7 cells were also co-transfected with increasing concentrations of HA-US28-WT and a luciferase-based reporter gene containing the VEGF gene promoter spanning region -1176/+54 (VEGF-Luc). As can been seen in Figure 3A, US28 induced a constitutive dose-dependent increase in VEGF promoter activation. The contribution of the constitutive activity of US28 and the involvement of chemokine binding were assessed by using the G protein-uncoupled mutant of US28, HA-US28-$R^{129}$A, which is still able to bind chemokines but is not capable of signaling through G proteins (Waldhoer, Casarosa et al. 2003), and the N-terminus-deleted mutant of US28, HA-$\Delta$2-22-US28, that can not bind chemokines but can still signal (Casarosa, Menge et al. 2003) (data not shown). Results showed that the constitutive activation of G proteins by US28, rather than the binding of endogenous chemokines, is crucial for VEGF promoter activation as the VEGF activation was completely abrogated for US28-$R^{129}$A (Figure 3B). By inducing VEGF promoter activation, US28 might constitutively induce angiogenic processes, essential for an oncogenic phenotype.

### US28 activates VEGF promoter via $G\alpha_q$, $G\beta\gamma$, p38 and p44/42 kinases

Unlike cellular chemokine receptors, US28 couples besides to $G\alpha_{i/o}$, also to $G\alpha_q$, known to activate proliferative signaling pathways (Radhika and Dhanasekaran 2001). In order to determine which signaling pathways are activated by US28 leading to VEGF gene expression, we assessed the involvement of $G\alpha_{i/o}$, $G\alpha_q$ and $G\beta\gamma$ proteins, as well as the role of mitogen-activated protein kinases (MAPK), using pertussis toxin (PTX), co-transfection with $G\alpha_q$ and $G\beta\gamma$ scavengers and kinase inhibitors, respectively.

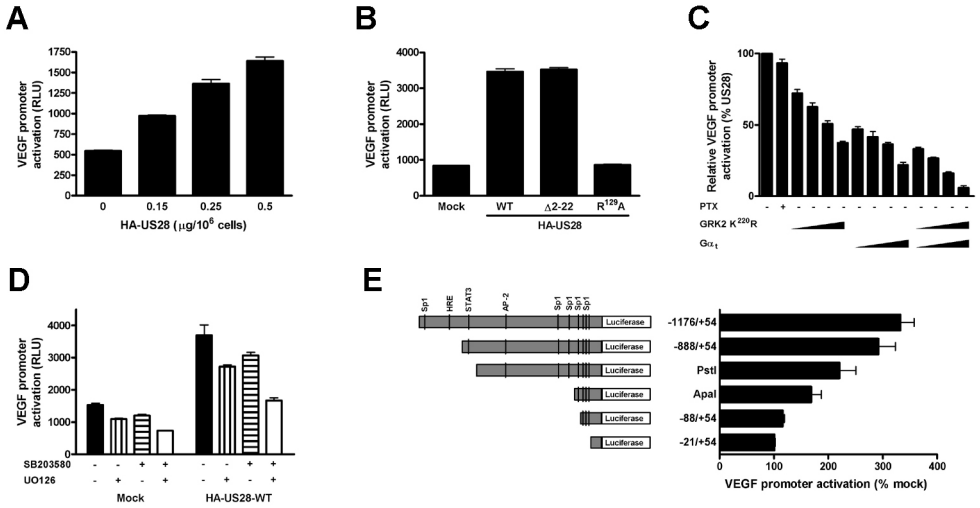


**Figure 3.** US28 constitutively activates VEGF promoter. **A**, In transiently transfected COS-7 cells, HA-US28-WT induced a dose-dependent constitutive activation of the VEGF gene promoter. **B**, In the VEGF-reporter gene assay, only the N-terminus deleted D2-22-US28 mutant induced a VEGF promoter activation similar to the WT receptor, whereas the G protein-uncoupled US28-R129A mutant showed no VEGF promoter activation. **C**, G protein coupled receptor kinase 2 dominant negative GRK2-K220R and Gatransducin (Gat) were co-transfected in different ratios with US28 (scavenger:US28 ratios were 0.5, 1, 2 and 4) into COS-7 cells and showed a dose-dependent VEGF inhibition, involving both Gaq/11 and bg subunits. **D**, The use of kinases inhibitors U0126 (1mM) and SB203580 (2mM) revealed that respectively mitogen-activated protein kinase (MAPK) p44/42 and p38 are independently involved in US28-mediated VEGF gene promoter activation. **E**, VEGF promoters of different length possess different binding sites for transcription factors (activator protein-2 AP-2, stimulating protein 1 Sp1, hypoxia inducible factor-1 responsive element HRE, signal transducer and activator of transcription 3 STAT3). The US28-mediated VEGF promoter activation was related to the length of the promoters, highlighting the involvement of transcription factors such as the hypoxia inducible factor-1 (HIF-1), STAT3, AP-2 and Sp1.

Our results indicated that US28-mediated VEGF activation is PTX-insensitive, showing that $G\alpha_{i/o}$ proteins are not involved in US28-mediated VEGF gene expression (Figure 3C). By co-transfecting the dominant negative of G protein-coupled receptor kinase 2 (GRK2) $K^{220}R$ or $G\alpha_{transducin}$ ($G\alpha_t$), known to scavenge both $G\alpha_{q/11}$ and $G\beta\gamma$ or only $G\beta\gamma$ respectively, we clearly observed a dose-dependent inhibition of VEGF activation. When both scavengers were over-expressed together, the inhibition was almost complete confirming the involvement of both $G\alpha_q$ and $G\beta\gamma$ subunits (Figure 3C). Using specific inhibitors of the protein kinases p38 and p44/42 MAPK, respectively SB203580 and U0126, US28-mediated VEGF promoter activation could be inhibited to a great extent (Figure 3D). When using both inhibitors, both pathways showed

to be independently involved, as measured by enhanced inhibition. To determine the involvement of various transcription factors in the US28-mediated VEGF gene promoter activation, we used different deletion mutants of the VEGF luciferase reporter gene (Legros, Bourcier et al. 2004). Upon truncation of the VEGF promoter, the US28-mediated luciferase activation decreased to be completely abolished when the promoter reached a minimum length (spanning region -27/+54). As can be seen in Figure 3E, the hypoxia inducible factor-1 (HIF-1), signal transducer and activator of transcription 3 (STAT3), activator protein-2 (AP-2) and stimulating protein 1 (Sp1) were important transcription factors involved in the US28-induced VEGF promoter activation. Our results indicate that US28 employs $G\beta\gamma$ and $G\alpha_{q/11}$ proteins, and the protein kinases p38 and p44/42 MAPK to activate downstream transcription factors including HIF1, STAT3, AP-2 and Sp1 to induce VEGF promoter activation.

## US28 promotes tumor formation *in vivo*

Since our *in vitro* studies showed that US28 induced a transformed and pro-angiogenic phenotype, we determined whether US28-WT expressing cells could also induce tumor formation *in vivo* (Figure 4A). To this end, stably transfected NIH-3T3 cells were subcutaneously injected in both flanks of nude mice (8 mice injected per cell line, 16 inoculations). First signs of tumor formation appeared as early as one week post-injection for the US28-WT group. The presence of tumors was obvious 2 weeks after inoculation. (Figure 4B). All the US28-WT injected mice presented tumors at all inoculation sites 3 weeks after injection, after which they were sacrificed. At this time point, both the mock and the US28-$R^{129}$A groups did not show any tumor formation (Figure 4A). However, 6 weeks post-injection, the US28-$R^{129}$A group started showing tumors. These tumors grew at a slower rate compared to the US28-WT group and they did not appear at all inoculated sites (81% take rate for the US28-$R^{129}$A group) (Figure 4B). The mock group did not develop any tumor, even as long as 75 days after injection. As a control, gene expression of US28 was confirmed by RT-PCR in all the tumors formed (Figure 4C). These data indicate that the constitutive activation of G proteins by US28 is a key player in the early onset of tumor formation. However, non G protein signaling pathways might also contribute to the tumorigenic properties of this receptor.

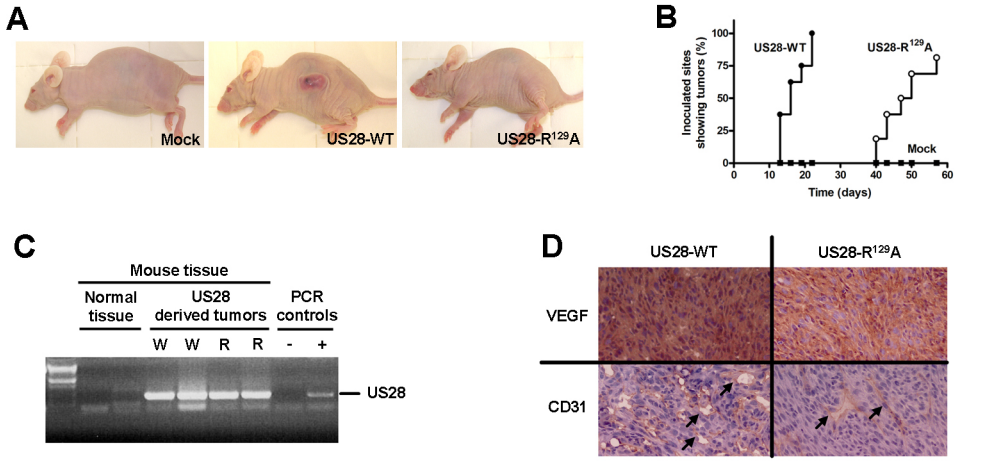


**Figure 4.** US28 promotes tumor formation *in vivo*. **A**, nude mice were injected with mock, US28-WT and US28-$R^{129}$A expressing NIH-3T3 cells. 3 weeks after inoculation of the cells, the US28-WT group was the only group showing extensive tumor formation. **B**, Kaplan-Meier curves presenting inoculation sites showing tumor formation in the mock (filled square), US28-WT (filled circle) and US28-$R^{129}$A (empty circle) groups (8 mice, 16 inoculation sites). The US28-$R^{129}$A group showed delayed tumor formation compared to the US28-WT, and the take rate was also lower (81% versus 100%). The mock group showed no tumor formation during the 75 days observation period. **C**, PCR showing the presence of US28 DNA in mice injected with NIH-3T3 cells expressing US28-WT (W) and US28-$R^{129}$A (R). **D**, VEGF expression (red) and the formation of new blood vessels (indicated by the arrow) with CD31 staining (red) in the formed tumors.

Since the tumors appeared highly vascularized and US28-WT induced the production of VEGF *in vitro*, we investigated the VEGF plasma levels in the three different groups. 3 weeks after inoculation, the VEGF plasma levels in the mock, US28-WT and US28-$R^{129}$A groups were respectively 61 ± 18, 140 ± 40 and 83 ± 23 pg/ml, showing that US28-WT constitutive activity had led to an increase in the VEGF plasma level, which might explain the earlier and stronger onset of tumorigenesis. In addition, tumors of both US28-WT and US28-$R^{129}$A groups stained positive for the presence of VEGF (Figure 4D, top panel), showing that VEGF might have a direct role on the site of the tumor, most likely by inducing angiogenesis. In order to check for the presence of newly formed blood vessels, immunostaining against CD31 was performed. As shown on Figure 4D (bottom panel), all tumors (US28-WT and US28-$R^{129}$A) stained positive for CD31, confirming the angiogenic processes in the tumors induced by both receptors, WT and mutant. The *in vivo* experiment clearly demonstrated the tumorigenic properties of US28 that were accompanied by VEGF secretion and formation of new blood vessels within the tumor.

## US28 is responsible for HCMV-induced angiogenic phenotype

In order to determine the importance of US28 in the viral context, we evaluated the impact of the deletion of the US28 gene in a newly established clinical HCMV strain (Titan). The virus was isolated from an infected patient according to the technique of Borst et al. (Borst, Hahn et al. 1999). The US28 deletion mutant of HCMV (HCMV-ΔUS28) showed neither US28 transcription (Figure 5A) or binding of CCL5 (Figure 5B). As HCMV has been shown to induce the production of VEGF (Reinhardt, Schaarschmidt et al. 2005) and it has been linked to the development of glioma (Cobbs, Harkins et al. 2002), we studied the effect of HCMV-WT and HCMV-ΔUS28 on VEGF regulation in the HCMV-permissive glioblastoma cell line U373.

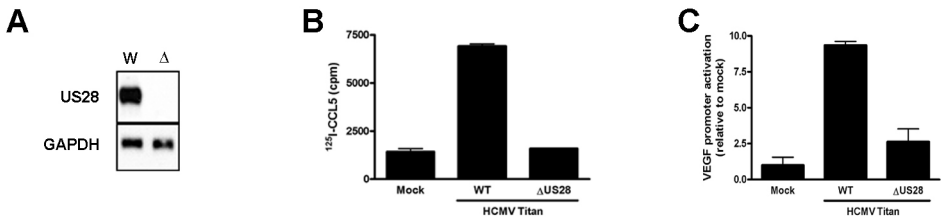


**Figure 5.** US28 is involved in HCMV-induced VEGF upregulation. **A**, northern blot analysis on infected human foreskin fibroblasts (HFF) with HCMV strain Titan WT (W) or the ΔUS28 deletion mutant (Δ) showed transcription of US28 gene only for the WT strain. Both infected cells showed similar GAPDH levels. **B**, $^{125}$I-CCL5 binding showing that US28 was expressed on the surface of HFF cells infected with HCMV WT, but not with the ΔUS28 deletion mutant. **C**, U373 cells infected with HCMV-WT or the HCMV deletion mutant HCMV-ΔUS28 showed that US28 plays a major role in the VEGF gene promoter activation in infected cells.

Infection of U373 cells showed that HCMV-WT strongly activated the VEGF promoter (Figure 5). This activation was severely attenuated with the deletion mutant HCMV-ΔUS28 and it was not significantly different from the activation in mock infected cells ($p>0.05$). These data indicate that US28 plays a crucial role in the VEGF promoter activation in HCMV-infected cells.

## Discussion

It is well established that some DNA viruses induce oncogenesis. Human papilloma virus (HPV) and KSHV e.g. are the etiological agent of respectively cervical cancer (Munoz, Bosch et al. 2003) and Kaposi's sarcoma (Ganem 1997); and Epstein-Barr virus (EBV) is associated with Burkitt's lymphoma and Hodgkin's disease (Young and Rickinson 2004). Unlike these oncogenic viruses, HCMV infection fails to transform susceptible normal cells. Yet, in tumor cells, as observed for oncoproteins of HPV types 16 and 18, and human adenovirus, some HCMV-encoded proteins possess oncomodulatory properties, modulating key signaling pathways thereby promoting tumor cell proliferation (Cinatl, Vogel et al. 2004). Interestingly, HCMV, like KSHV, encodes a viral chemokine receptor, that unlike its cellular homologue binds a broad spectrum of chemokines and displays constitutive activity (Vischer, Leurs et al. 2006). The KSHV-encoded receptor ORF74 has previously been shown to act as a viral oncogene, inducing angioproliferative lesions that morphologically resemble Kaposi's sarcoma (Yang, Chen et al. 2000). In this study we demonstrate for the first time that the HCMV-encoded chemokine receptor US28 might act as a viral oncogene. Expression of US28 in NIH-3T3 cells induces transformation and promotes tumorigenesis *in vivo*, in part by activating pro-angiogenic signaling pathways. In non-tumorigenic cells, however, we have recently shown that US28 induces apoptosis (Pleskoff, Casarosa et al. 2005), indicating that the oncomodulatory properties of US28 are only apparent when cells present a tumorigenic phenotype or are on the verge of transformation, such as NIH-3T3 cells. A hallmark of the viral-encoded chemokine receptors is their ability to signal in a constitutively active manner (Vischer, Leurs et al. 2006). This property appears essential in the early onset of tumorigenesis induced by US28, as shown by the delayed and attenuated tumor formation by the US28-$R^{129}A$ mutant. Although the mutation in the DRY motif of US28-$R^{129}A$ prevents G protein activation, some residual activity such as a slightly enhanced proliferation index and increased expression of cyclin D1 might account for the tumor formation in mice injected with US28-$R^{129}A$ expressing cells. As shown for other receptors (Rajagopal, Lefkowitz et al. 2005), US28 might also use G protein-independent signaling pathways to exert its oncogenic potential. In particular since the US28-$R^{129}A$ mutant is not devoid of chemokine binding, chemokines may activate US28, stimulating non-G protein signaling pathways.

Also in infected U373 cells, HCMV induced VEGF promoter activation. When using the newly developed clinical HCMV US28 deletion strain, this VEGF promoter activation was not apparent, indicating that US28 is essential for the angiogenic phenotype observed after viral infection. As

such, after HCMV infection US28 might act in a concerted manner with other HCMV-encoded proteins, which were previously linked to oncogenesis, such as e.g. the viral homologue of interleukin-10 (cmvIL-10) (Doniger, Muralidhar et al. 1999; Kotenko, Saccani et al. 2000) and immediate-early proteins (Castillo and Kowalik 2002). The constitutive activity of US28 and its ability to bind chemokines, known to be markedly expressed in certain types of cancer (Balkwill 2004), might facilitate progression of tumor formation after infection. In particular, reactivation of HCMV in immunocompromised cancer patients, might boost expression of US28, further promoting the oncogenic potential of HCMV. In view of its tumorigenic properties, US28 can be regarded as a potential drug target for the treatment of HCMV-related proliferative diseases.

## Materials and Methods

**Cell culture.** African green monkey COS-7 cells, human glioblastoma U373 cells and mouse fibroblast NIH-3T3 cells were cultured in DMEM supplemented with 10% of fetal calf, heat inactivated fetal calf and calf sera, respectively. Transfections were performed in COS-7 using the DEAE-Dextran method (Casarosa, Bakker et al. 2001), and in U373 and NIH-3T3 cells using the calcium phosphate method. Stably transfected NIH-3T3 cells were selected and maintained in culture with neomycin (400 μg/ml) to ensure homogenous expression of US28 receptors.

**US28 receptor characterization and thymidine incorporation.** US28 expression and constitutive signaling were checked using $^{125}$I-CCL5 binding (aspecific binding was measured using Fraktalkine/CX3CL1 $10^{-7}$M) and $^{3}$H-inositol phosphate formation as previously described(Casarosa, Bakker et al. 2001). As for thymidine incorporation measurement, the experiment was carried out upon serum starvation using medium containing 0.5% calf serum (Westphal and Sanders-Bush 1996).

**Reporter gene analysis.** The VEGF reporter gene plasmids were composed of different lengths of the VEGF promoter, hence containing different binding sites for transcription factors as previously described (Legros, Bourcier et al. 2004). For the VEGF promoter activation measurements, $10^6$ COS-7 cells were transfected with a 5 μg of pGL2-VEGF-Luciferase plasmid and the indicated amounts (or 0.5 μg when not stated) of pcDEF$_3$-HA-US28 receptors (wild type, G protein-uncoupled mutant R$^{129}$A and N-terminus deleted □2-22). When using inhibitors or G protein scavengers, they were respectively added or co-transfected together with US28 (total DNA amounts were kept constant using empty vector). In U373 infected cells, transfection of the VEGF-Luciferase plasmid (Finkenzeller, Sparacio et al. 1997) was performed 2 hours post infection (M.O.I. 1). Luciferase activities were measured 24h post transfection.

**Focus formation assay.** The focus formation assay was performed as described by Burger et al (Burger, Burger et al. 1999). Stably transfected NIH-3T3 cells (2 x $10^3$) were cultured with $2.10^5$ untransfected NIH-3T3 cells for 2 weeks in regular culture medium without G418.

**VEGF ELISA.** VEGF amounts released by stably transfected NIH-3T3 cells after 5 days of culture or VEGF present in the plasma of the mice from the *in vivo* study were measured using a mouse-VEGF Quantikine kit (R&D), following the manufacturer's recommended procedures.

**Tumor formation *in vivo*.** All animal experiments were performed according to National Institutes of Health principles of laboratory animal care and Dutch national law ("Wet op de Dierproeven", Stb 1985, 336) and approved by the Dierexperimentencommissie from the VU Medical Centre and performed in compliance with the protocol FaCh 05-02. Stably transfected NIH-3T3 cells ($2.10^6$) containing pcDEF$_3$, pcDEF$_3$-US28-WT or pcDEF$_3$-US28-R$^{129}$A plasmids were injected subcutaneously in the flank of 8-10 old weeks female nude mice (Hsd, athymic *nu/nu*, 25-32 g, Harlan/CPB, Zeist, The Netherlands).

**Immunohistochemitry.** Cryosections of the US28-WT and US28-R$^{129}$A tumors were stained for the presence of VEGF and CD31 using the following antibodies: goat anti-mouse VEGF antibody (AF-493-NA, R&D Systems, 10 μg/ml) with a rabbit-anti-goat-HRP (P 0449, Dako cytomaton, 1:100), and rat-anti-mouse CD31 (550274, BD Pharmingen Erembodegem, 1:10) with a mouse-anti-rat-HRP (80-9520, Zymed Laboratories, 1:100). Nuclear staining was performed with Haematoxylin (Merck).

**RT-PCR for US28.** In the tumors formed, US28 gene expression was checked using standard reverse transcriptase polymerase chain reaction (RT-PCR). The primers used were US28 forward 5'-AGCGTGCCGTGTACGTTAC-3' and US28 reverse 5' -ATAAAGACAAGCACGACC-3'.

**Cell cycle analysis.** Stably transfected NIH-3T3 cells were synchronized for 24h in DMEM containing 0.5% calf serum, and stimulated using 10% calf serum containing DMEM for another 24h. Cells were stained with propidium iodine and cell cycle populations were determined using the Guava EasyCyt system according to the manufacturer's recommendations (Guava). The Guava Cell Cycle™ software was used to determine the cell populations in the different cell cycle phases and the proliferation index (P.I.) was quantified from the $SG_2M/G_0G_1$ ratios.

**Western Blot analysis.** Quantification of the Cyclin D1 expression levels were performed by Western Blot on total cell lysates using a monoclonal mouse Cyclin D1 antibody (05-815, Upstate, 1μg/ml). Protein expression levels were related to □-actin expression (A5441, Sigma, 1:10,000).

**Cytomegalovirus strains creation.** The Titan strain was generated from a low passaged clinical isolate by the BAC-technique of Borst et al. (Borst, Hahn et al. 1999) and the US28-deletion mutant was created by the ET-recombinant method according to Wagner and Koszniwski (Wagner and Koszinowski 2004). The HCMV-WT and □US28 strains were characterized by Northern Blot and $^{125}$I-CCL5 binding as previously described (Minisini, Tulone et al. 2003).

**Statistical analysis.** All *in vitro* experiments were performed at least three times in triplicates. When different groups or cell lines were compared, one-way ANOVA analysis were performed using a Tukey post-test with the GraphPad Prism software. Bars and error bars on the graphs as well as data in the text represent the mean ± s.e.m.

# IV

# The human cytomegalovirus-encoded chemokine receptor US28 promotes angiogenesis and tumor formation via cyclooxygenase-2

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*D.M. and E.L. contributed equally to this work.

Adapted from Maussang, Langemeijer et al., *Cancer Res*, 2009, 69(7): 2861-9.

**Abstract**

The human cytomegalovirus (HCMV), potentially associated with the development of malignancies, encodes the constitutively active chemokine receptor US28. Previously, we have shown that US28 expression induces an oncogenic phenotype both *in vitro* and *in vivo*. Microarray analysis revealed differential expression of genes involved in oncogenic signaling in US28-expressing NIH-3T3 cells. In particular, the expression of cyclooxygenase-2 (COX-2), a key mediator of inflammatory diseases and major determinant in several forms of cancer, was highly upregulated. US28 induced increases in COX-2 expression via activation of NF-κB, driving the production of vascular endothelial growth factor (VEGF). Also in HCMV-infected cells, US28 contributed to the viral induction of COX-2. Finally, the involvement of COX-2 in US28-mediated tumor formation was evaluated using the COX-2 selective inhibitor Celecoxib. Targeting COX-2 *in vivo* with Celecoxib led to a marked delay in the onset of tumor formation in nude mice injected with US28-transfected NIH-3T3 cells and a reduction of subsequent growth by repressing the US28-induced angiogenic activity. Hence, the development of HCMV-related proliferative diseases may partially be ascribed to the ability of US28 to activate COX-2.

## Introduction

Herpesviruses are widespread pathogens, which establish a life-long latent and persistent infection. In immunocompetent hosts, infection is often asymptomatic while reactivation can lead to serious pathological conditions (Rosenkilde, Smit et al. 2008). In particular, gamma herpesviruses possess oncogenic potential, as they are able to transform cells upon infection (Flore, Rafii et al. 1998). Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi's sarcoma (Ganem 1997), whereas Epstein-Barr virus (EBV) is associated with lymphoproliferative diseases such as Burkitt's lymphoma and Hodgkin's disease (Young and Rickinson 2004). Unlike KSHV and EBV, the human cytomegalovirus (HCMV) is not considered an oncogenic herpesvirus (Damania 2004), but it has been suggested to act as an oncomodulator (Cinatl, Vogel et al. 2004). The presence of HCMV proteins has been detected in several malignancies, such as colon cancer (Harkins, Volk et al. 2002), malignant glioblastoma (Sabatier, Uro-Coste et al. 2005) and breast cancer (Soderberg-Naucler 2008). Importantly, HCMV preferably infects tumor cells, leading to enhanced cell proliferation, angiogenesis and resistance to apoptosis (Cinatl, Vogel et al. 2004; Michaelis, Kotchetkov et al. 2004).

Of interest, most herpesviruses contain one or more genes that encode for constitutively active G-protein coupled receptors (GPCRs) (Rosenkilde, Smit et al. 2008). These viral GPCRs show highest homology to the class of chemokine receptors, known to be involved in the control of the immune system but also the development of various types of cancer (Balkwill 2004). As such, these viral GPCRs likely contribute to viral pathogenesis. The KSHV-encoded GPCR ORF74 is believed to act as a viral oncogene and is considered a key determinant in the pathology of Kaposi's sarcoma. ORF74 possesses proliferative, angiogenic and anti-apoptotic properties, and drives the cell transforming properties of KSHV (Bais, Santomasso et al. 1998; Yang, Chen et al. 2000). Recently, we have shown that the HCMV-encoded chemokine receptor US28, which can bind several chemokines (e.g. CCL2, CCL5, CX3CL1), constitutively activates various inflammatory and proliferative signaling pathways such as NF-κB and induces tumor formation *in vivo* (Maussang, Verzijl et al. 2006). Expression of US28 activates $G\alpha_q$-linked signaling pathways, e.g. production of inositol phosphate (Casarosa, Bakker et al. 2001), resulting in an increase in cyclin D1 expression, DNA synthesis and secretion of vascular endothelial growth factor (VEGF) (Maussang, Verzijl et al. 2006). Mice injected with US28-expressing NIH-3T3 cells develop tumors with high VEGF expression. Moreover, an increase in VEGF promoter activity is

also apparent in HCMV-infected glioblastoma cells, which can be attributed to the expression of US28 (Maussang, Verzijl et al. 2006). Interestingly, US28 expression has also been detected during primary and secondary HCMV infection in immunosuppressed patients (Boomker, Verschuuren et al. 2006). Considering the pathogenic potential of HCMV in these patients, US28 expression at this stage may play a role in the progression of HCMV-linked proliferative diseases.

Profiling of the expression of thousands of genes by means of DNA microarrays has served to discover new oncogenes and potentially new targets for the treatment of cancer (Russo, Zegar et al. 2003). It has also been used to understand molecular mechanisms underlying the development of herpesvirus associated diseases (Zhu, Cong et al. 1998; Moses, Jarvis et al. 2002; Schlee, Holzel et al. 2007). To gain mechanistic insight into the oncogenic behavior of the HCMV-encoded chemokine receptor US28, we performed microarray analysis on US28 and mock-transfected cells. Various proteins involved in oncogenesis were found to be modulated by the expression of US28. In particular, cyclooxygenase-2 (COX-2) was highly upregulated upon US28 expression. Also in HCMV-infected cells, US28 contributed to the viral induction of COX-2. Finally, the COX-2 specific inhibitor Celecoxib did not only inhibit the upregulation of VEGF in US28-expressing cells, but also markedly decreased US28-induced tumor formation rate in nude mice. As such, US28 upregulates COX-2 expression to promote tumor formation.

## Results

### Microarray analysis of US28-expressing cells

Expression of the HCMV-encoded chemokine receptor US28 in NIH-3T3 cells induces increased cell growth and a pro-angiogenic phenotype (Maussang, Verzijl et al. 2006). To gain insight into the underlying mechanisms of US28-related oncogenic transformation, we analyzed gene expression profiles of US28-expressing NIH-3T3 cells and the corresponding mock-transfected NIH-3T3 cells by cDNA microarray analysis. US28 wild type (WT)-expressing cells showed increases in [$^{125}$I]-CX3CL1 binding (Figure 1A) and inositol phosphate production (Figure 1B), which was not apparent in mock-transfected cells. To reproducibly identify differentially expressed genes in our microarray analysis, two independent clonal cell lines, with similar receptor expression and functional characteristics (Figure 1A and B) were used for both mock and US28-transfected NIH-3T3 cells. Analysis of the overall microarrays intensities showed highest correlation between biological duplicates (i.e. mock to mock, US28 to US28) (data not shown).

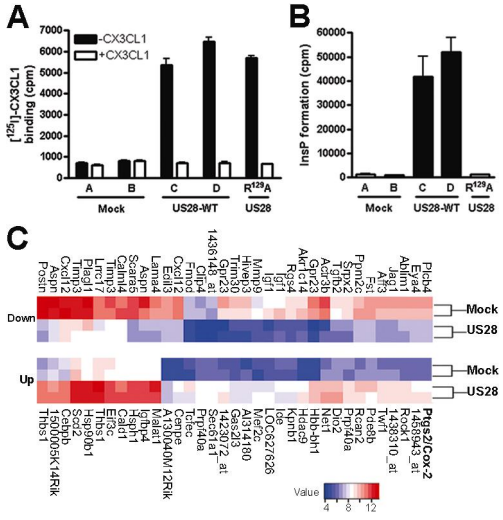


**Figure 1**. Characterization and microarray analysis of US28-expressing NIH-3T3 cells. **A**, independent stable clonal NIH-3T3 cell lines transfected with US28-WT and the G-protein uncoupled mutant US28-R129A bind [125I]-CX3CL1. Unlabelled CX3CL1 displaces specifically bound [125I]-CX3CL1. **B**, US28-WT constitutively induces the formation of inositol phosphate (InsP), whereas US28-R129A does not. **C**, Affymetrix Mouse Genome Array data from US28 and mock-transfected cells were analyzed with the LIMMA software using a false discovery rate £ 0.02. The 35 most upregulated (Up) and downregulated (Down) probe sets are represented with a heatmap. Colors indicate log2 intensity values of normalized probe sets.

Expression data of the 45,001 Affymetrix probe sets on the Mouse Genome 430 2A Array were normalized (RMA) and analyzed with LIMMA (Smyth 2005). Using a false discovery rate (FDR) lower or equal to 0.02, we obtained 577 probe sets representing 556 genes (Supplementary Table S1). The most highly modulated genes are depicted in Figure 1C. As can be seen in this figure, the observed

changes in gene expression were comparable in the two different clonal mock and US28-expressing cell lines. The involvement of differentially expressed genes in biological pathways was analyzed using DAVID (Dennis, Sherman et al. 2003) for testing KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (Kanehisa and Goto 2000). The 556 genes did not present statistically enriched signaling pathways (after Benjamini correction for multiple testing). Annotation of the genes to the KEGG pathways using the EASE software (Hosack, Dennis et al. 2003) highlighted pathways involved in focal adhesion, actin cytoskeleton regulation, cell cycle and several forms of cancer, but also signaling pathways such as p53, MAPK, Wnt, and TGFβ (Supplementary Table S2).

**Table 1.** LIMMA analysis of genes differentially regulated between mock and US28-expressing cells were obtained using a false discovery rate ≤ 0.02 (Supplementary Table S1). The retrieved gene list contained 16 cancer-related genes and 8 NF-kB transcriptional targets. Multiple fold change values represent multiple probe sets available on the array.

| Gene symbol | Gene Name | Fold change |
|---|---|---|
| **CancerGenes resource** | | |
| Net1 | Neuroepithelial cell transforming gene 1 | 11.5 |
| **Ptgs2/Cox-2** | Prostaglandin-endoperoxide synthase 2 | 6.6 |
| Kpnb1 | Karyopherin (importin) beta 1 | 6.1 |
| Cebpb | CCAAT/enhancer binding protein (C/EBP), beta | 10.9, 2.9, 2.2 |
| Actb | Actin, beta | 5.1, 3.5, 2.4 |
| Klf6 | Kruppel-like factor 6 | 4.1, 3.3 |
| Cttn | Cortactin | 3.4, 3.0 |
| Fus | Fusion (involved in t(12;16) in malignant liposarcoma) | 3.5 |
| Runx1 | Runt-related transcription factor 1 | 2.9 |
| Akt1 | V-akt murine thymoma viral oncogene homolog 1 | 2.7 |
| Araf | V-raf murine sarcoma 3611 viral oncogene homolog | 2.2 |
| Mdm2 | Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) | 2.2 |
| Gli3 | GLI-Kruppel family member GLI3 | -3.1 |
| Maf | V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian) | -3.1 |
| Timp3 | TIMP metallopeptidase inhibitor 3 | -6.3, -9.2 |
| Aff3 | AF4/FMR2 family, member 3 | -9.8 |
| **NFkB transcriptional targets** | | |
| **Ptgs2/Cox-2** | Prostaglandin-endoperoxide synthase 2 | 6.6 |
| Hgf | Hepatocyte growth factor | 4.9, 4.8, 3.1 |
| Serpine1 | Serine (or cysteine) peptidase inhibitor, clade E, member 1 | 4.2 |
| Nr4a2 | Nuclear receptor subfamily 4, group A, member 2 | 3.8, 3.6, 2.8 |
| Gadd45b | Growth arrest and DNA-damage-inducible 45 beta | 2.8 |
| Csf1 | Colony stimulating factor 1 (macrophage) | 2.6 |
| Cflar | CASP8 and FADD-like apoptosis regulator | 2.3 |
| C3 | Complement component 3 | -2.8 |
| Mmp9 | Matrix metallopeptidase 9 | -2.8, -6.8 |
| Sox9 | SRY-box containing gene 9 | -2.9 |

To focus on molecular mediators that contribute to the oncogenic potential of US28, we used the *CancerGenes* resource (Higgins, Claremont et al. 2007), identifying amongst others, Akt1, Fus, Klf6, Mdm2 and Ptgs2/Cox-2 (Table 1). In addition, since US28 has previously

been shown to constitutively activate NF-κB signaling pathways (Casarosa, Bakker et al. 2001), the list of modulated genes was compared to known transcriptional targets of NF-κB (adapted from http://jura.wi.mit.edu/young_public/nfkb/literature_targets.xls) (Table 1). Ptgs2/Cox-2 also appeared to be one of the most strongly upregulated NF-κB target genes. This finding of Ptgs2/Cox-2 in both cancer and NF-κB gene lists was of particular interest since COX-2 protein is highly upregulated in various forms of cancers (Turini and DuBois 2002), but also induced upon HCMV-infection (Zhu, Cong et al. 1998).

To validate the microarray expression data, a few genes with high fold changes were analyzed by means of quantitative RT-PCR (qPCR). As observed in the microarray experiment, Ptgs2/Cox-2 as well as other highly differentially expressed genes (Mef2c, Cxcl12 and Tgfb2) showed a similar degree of up or downregulation upon expression of US28 in NIH-3T3 cells (Table 2). Because of the previously reported oncogenic potential of US28 *in vivo*, we also determined expression levels of these genes in RNA extracted from 5 independent US28-induced tumors derived from our xenograft model (Maussang, Verzijl et al. 2006). Expression of US28 was confirmed in all mouse tumors by qPCR and Ptgs2/Cox-2 mRNA was highly upregulated in US28-induced tumors, highlighting a potential important role for COX-2 during tumorigenesis.

| Modu-lation | Gene symbol | Microarray US28 NIH-3T3 cells | qPCR US28 NIH-3T3 cells | qPCR US28 mice tumors |
|---|---|---|---|---|
| Up | Us28 | | 2860 ± 694 | 475 ± 45 |
| | Mef2c | 8.9, 2.0, 2.2 | 5.2 ± 1.2 | 5.5 ± 0.7 |
| | Ptgs2/Cox-2 | 6.6 | 15.8 ± 4.8 | 22.3 ± 6.3 |
| Down | Cxcl12 | 10.8, 12.2 | 33.7 ± 3.1 | 416.9 ± 96.8 |
| | Tgfb2 | 6.4 | 35.3 ± 1.4 | 238.3 ± 34.0 |

**Table 2.** Validation of microarray expression data by qPCR in US28-stably transfected NIH-3T3 cells and US28-derived mouse tumors (n=5). Values represent average ± s.e.m. of the fold change in US28-derived samples (cells or tumor) vs mock-transfected cells. Up, upregulated genes; down, downregulated genes.

## US28 constitutive activity upregulates COX-2 expression

COX-2 is highly upregulated in a variety of cancers and is known to drive expression of cyclin D1 and VEGF (Dorsam and Gutkind 2007). Since COX-2 is also upregulated in HCMV-infected cells (Zhu, Cong et al. 1998) and expression of US28 results in induction of cyclin D1 and VEGF expression (Maussang, Verzijl et al. 2006), we decided to further focus

on COX-2 and examine its role in US28-induced proliferative signaling and tumor formation. US28-WT-expressing cells, but not cells expressing the G-protein uncoupled mutant US28-$R^{129}$A, have been shown to present a transformed phenotype *in vitro* (Maussang, Verzijl et al. 2006). NIH-3T3 cells expressing US28-$R^{129}$A showed comparable receptor expression levels to US28-WT-expressing cells as measured by [$^{125}$I]-CX3CL1 binding (Figure. 1A), but did not show increases in inositol phosphate accumulation (Figure 1B). Analysis of COX-2 mRNA expression by qPCR showed a 19.7 ± 1.8 fold increase in US28-WT-expressing cells compared to mock-transfected cells (Figure 2A). Cells expressing US28-$R^{129}$A revealed no significant difference (1.6 ± 0.6 fold) in COX-2 mRNA levels compared to mock-transfected cells (Figure 2A). Similarly, US28-WT-transfected NIH-3T3 cells showed a marked increase in COX-2 protein expression compared to mock-transfected and US28-$R^{129}$A-expressing cells (Figure 2B).

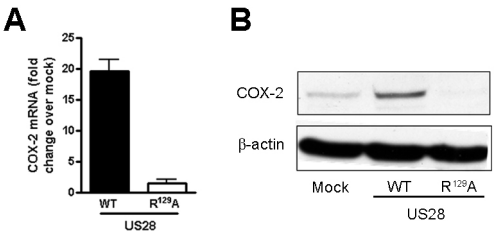


**Figure 2.** US28 constitutively upregulates COX-2 expression. ***A***, US28-WT-expressing NIH-3T3 cells (filled bar) present higher COX-2 mRNA levels than mock-transfected cells. US28-$R^{129}$A-expressing cells (open bar) do not show any modulation of COX-2 mRNA expression. ***B***, COX-2 protein expression is higher in US28-WT-expressing cells than in mock- and US28-$R^{129}$A-transfected cells.

## US28 induces COX-2 and VEGF transcription via activation of NF-κB

In order to understand the molecular mechanisms resulting in the upregulation of COX-2, signaling studies with a COX-2 promoter reporter (Iniguez, Martinez-Martinez et al. 2000) were performed in HEK 293T cells. US28 induced the human COX-2 promoter activation in a dose-dependent manner, but no increase in COX-2 promoter activity was observed in US28-$R^{129}$A-expressing HEK 293T (Figure 3A). Since the transcription of the COX-2 gene is under the control of NF-κB (Smith, DeWitt et al. 2000), we investigated the contribution of NF-κB in the COX-2 promoter reporter gene. US28-WT, but not US28-$R^{129}$A, constitutively activated the NF-κB transcription factor in transfected HEK 293T cells (Figure 3A). Moreover, inhibition of NF-κB activation with the IκB phosphorylation inhibitor Bay 11-7082 (5 μM) resulted in a severe reduction of US28-induced COX-2 promoter activation (Figure 3B). To further assess the role of NF-κB in the US28-induced COX-2 expression,

we used two truncated COX-2 promoter reporter genes that do not contain sequential NF-κB binding sites (Iniguez, Martinez-Martinez et al. 2000). 5'-deletions of the distal and both distal and proximal NF-κB binding sites reduced the US28-induced constitutive activation of the COX-2 promoter by respectively 30 and 80% compared to the non-deleted promoter (Figure 3C). Since COX-2 is known to drive expression of VEGF (Dorsam and Gutkind 2007), shown to be upregulated upon expression of US28 (Maussang, Verzijl et al. 2006), we investigated the role of NF-κB in VEGF promotor activity using the NF-κB inhibitor Bay 11-7082. As seen for the US28-induced COX-2 promotor activity, the US28-induced VEGF promoter activation was markedly inhibited by Bay 11-7082, highlighting the involvement of NF-κB in this process (Figure 3D).

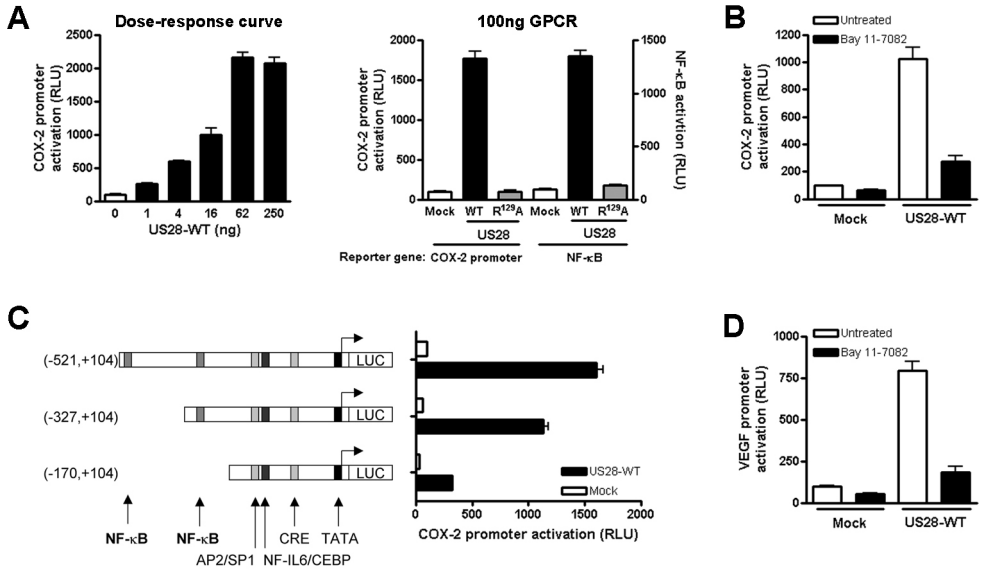


**Figure 3.** US28 activates the COX-2 and VEGF promoters via NF-κB. **A**, HEK 293T cells transiently transfected with increasing amounts of US28 show a dose-dependent activation of the human COX-2 promoter luciferase reporter gene. The human COX-2 promoter and the transcription factor NF-κB are activated after transfection of US28-WT, but not with the G-protein uncoupled mutant US28-$R^{129}A$. **B**, US28-WT-induced COX-2 promoter activation is inhibited by the NF-κB inhibitor Bay 11-7082 (5μM). **C**, the US28-WT-induced COX-2 promoter activity is reduced when one and in particular both NF-κB binding sites are removed in 5'-deletion mutants of the human COX-2 promoter. **D**, US28-WT-induced VEGF promoter activation is inhibited by the NF-kB inhibitor Bay 11-7082 (5μM).

## US28 induces COX-2 expression in HCMV-infected cells

Infection of cells with HCMV is associated with the activation of various signaling pathways linked to inflammation, including increased expression of COX-2 (Zhu, Cong et al. 2002). Since US28 induced COX-2 expression in transfected cells, we examined the role of US28 in a viral setting. To this end, we used a HCMV deletion virus derived from the AD169 strain that does not contain the sequence encoding for US28 (AD169-ΔUS28). Human foreskin fibroblasts were infected with either mock, AD169-WT or the deletion mutant AD169-ΔUS28 (M.O.I. 3). 8h post-infection (p.i.), only cells infected with the WT virus presented expression of US28 mRNA (Figure 4A). As expected, the deletion mutant (Δ) did not induce the expression of US28 mRNA. Radioligand binding studies demonstrated that also on the protein level, only cells infected with the AD169-WT virus expressed US28 on their cell surface (Figure 4B).

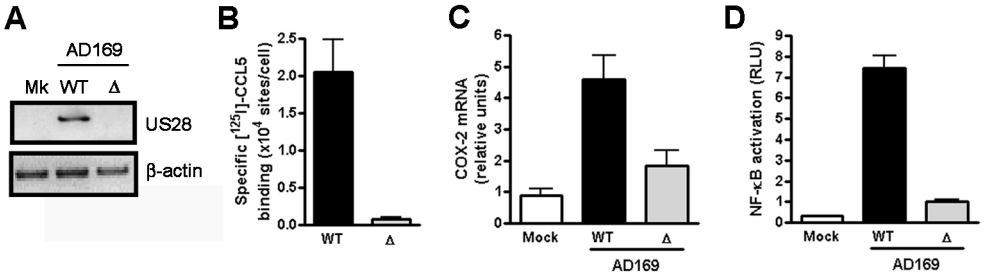


**Figure 4.** US28 mediates COX-2 expression in HCMV-infected cells. **A**, human foreskin fibroblasts infected with mock (Mk), AD169-WT or the deletion mutant AD169-ΔUS28 (Δ) viruses only express US28 mRNA with the WT virus 8h p.i. **B**, [$^{125}$I]-CCL5 binding shows US28 protein expression in AD169-WT-infected cells, but not in cells infected with AD169-ΔUS28 mutant. **C-D**, cells infected with AD169-WT present a higher expression level of COX-2 mRNA (**C**) and an increased NF-κB activation (**D**) compared to mock-infected cells. Deletion of US28 gene from the viral genome impairs COX-2 expression and NF-κB transcriptional activity in infected cells.

In accordance with earlier findings (Zhu, Cong et al. 2002), COX-2 mRNA levels were clearly upregulated in cells infected with the WT virus 8 h p.i., compared to mock-infected cells (Figure 4C). In cells infected with AD169-ΔUS28, COX-2 mRNA levels were markedly reduced, highlighting a role for US28 in the viral induction of COX-2. The observed decrease in COX-2 mRNA in AD169-ΔUS28-infected cells was not attributed to impairment in viral IE expression of this mutant strain (data not shown), indicating that the viral replication abilities of both viruses were similar. Since US28 is able to constitutively activate NF-κB

(Casarosa, Bakker et al. 2001), we also tested whether this was the case in the HCMV context. Infection of cells with AD169-WT was clearly associated with increased activation of NF-κB as measured using a NF-κB reporter gene (Figure 4D). When infecting cells with the US28 deletion mutant, we found that the HCMV-induced NF-κB activation was markedly impaired compared to cells infected with the WT virus (Figure 4D). This indicates that upon HCMV infection, US28 activates NF-κB, regulating in turn expression of its transcriptional target COX-2.

## COX-2 plays an important role in US28-mediated tumor formation

We have previously shown that US28 upregulates VEGF expression in stably transfected NIH-3T3 cells (Maussang, Verzijl et al. 2006). Since VEGF expression is in part regulated by COX-2 (Dorsam and Gutkind 2007), we tested *in vitro* the effect of the COX-2 specific non-steroidal anti-inflammatory drug (NSAID) Celecoxib on the US28-induced secretion of VEGF. When culturing US28-expressing NIH-3T3 cells with Celecoxib (25 μM), VEGF secretion was significantly reduced (52 ± 2%, $p<0.001$) indicating the involvement of COX-2 in the US28-induced pro-angiogenic phenotype (Figure 5A). As Celecoxib was able to inhibit *in vitro* an important angiogenic factor involved in US28-induced tumor formation, it was also used *in vivo* to examine the contribution of COX-2 during tumorigenesis. Celecoxib treatment was started one day prior to the injection of US28-stably transfected NIH-3T3 cells into nude mice by feeding the animals with chow containing 1500 ppm Celecoxib (Ragel, Jensen et al. 2007). As previously described, all control mice injected with US28-expressing cells developed tumors larger than 50 $mm^3$ within 3 weeks. Yet, at this time point, Celecoxib-fed mice showed a severely delayed onset in tumor formation (Figure 5B). Developing tumors reached the size of 50 $mm^3$ between 21 and 39 days post-injection, while for the control group, tumor formation occurred between 14 and 21 days post-injection. Celecoxib also increased the tumor doubling time. Untreated mice injected with US28-expressing cells displayed an average tumor doubling time of 3.4 ± 0.3 days, whereas Celecoxib-treated mice presented a longer doubling time of 6.4 ± 0.4 days.

When xenografts had reached their maximum size and animals were sacrificed, no significant changes in VEGF mRNA levels were apparent between the untreated and Celecoxib-treated tumors (data not shown). To measure effects of Celecoxib on the angiogenic activity in US28-induced tumors, we examined CD31 expression by immunohistochemistry. Xenografts derived from stably transfected NIH-

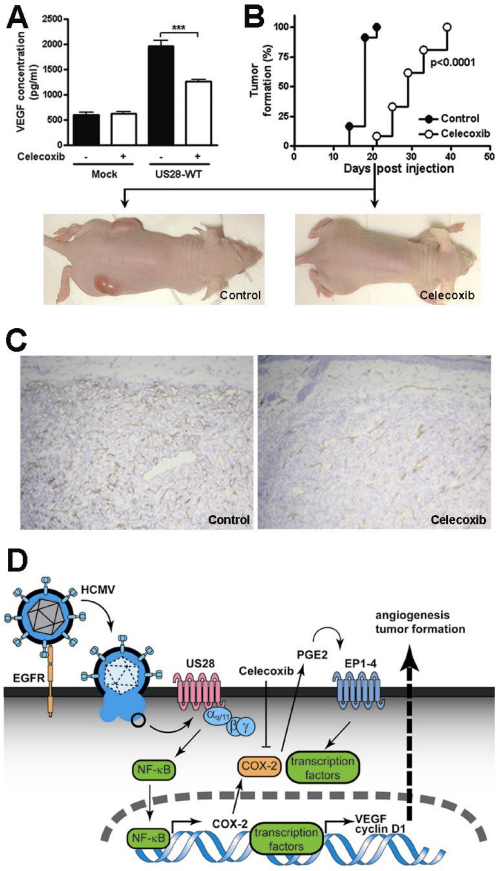


**Figure 5.** Celecoxib impairs US28 oncogenic potential. **A**, release of VEGF in the culture medium of US28-stably transfected NIH-3T3 cells is partly inhibited by Celecoxib (25 μM). **B**, Celecoxib-treated mice injected with US28-expressing cells (○) develop tumors bigger than 50 $mm^3$ later and at a slower rate than control mice (●). 20 days post-injection of US28-expressing cells into nude mice, control mice present extensive tumor formation while the Celecoxib-treated group (1500 ppm) is still devoid of tumors. **C**, representative CD31 staining in US28-induced tumors from control and Celecoxib-treated mice. **D**, schematic representation of US28-induced COX-2 upregulation and role during tumor formation. US28 is expressed at the cell surface of HCMV-infected cells and increases COX-2 expression via NF-kB. COX-2 activity leads to PGE2 release that can bind to its cognate receptors EP1-4 and subsequently induces VEGF and Cyclin D1 transcription. This results in angiogenesis and tumor formation that can be inhibited by Celecoxib. ***, $p<0.0001$.

3T3 cells lines show highest growth activity in the outer cell layers rather than at the center of the tumor, which can become necrotic when tumors become large. As expected, the angiogenic activity in the untreated US28-derived tumors was clearly apparent in the outer area of the malignancies with a dense localization of CD31 positive blood vessels (Figure 5C). Interestingly, the Celecoxib-treated tumors presented a lower staining intensity of CD31 and a less dense distribution of newly formed blood vessels compared to the untreated group. As such, COX-2 inhibition by Celecoxib delayed the onset of US28-induced tumors and slowed their overall growth by repressing angiogenic activity.

## Discussion

Infection of cells with herpesviruses is known to alter cellular gene expression and cell function. Functional genomic analyses of herpesvirus-infected cells have led to the identification of genes that contribute to viral pathogenesis, including induction of tumorigenic events (Zhu, Cong et al. 1998; Moses, Jarvis et al. 2002; Schlee, Holzel et al. 2007). In view of the reported oncomodulatory potential of HCMV (Cinatl, Vogel et al. 2004) and particularly the observed oncogenic properties of the HCMV-encoded chemokine receptor US28 (Maussang, Verzijl et al. 2006), we subjected US28-expressing cells to a detailed microarray analysis. As anticipated, US28 and mock-transfected NIH-3T3 cells differentially express genes involved in oncogenic events (e.g. renal cell carcinoma, prostate cancer and melanoma). Significantly modulated genes are implicated in cell cycle, p53 and MAPK pathways (Supplementary Table S2), all associated with proliferation (Toledo and Wahl 2006; Dhillon, Hagan et al. 2007; Roberts and Der 2007; Scian, Carchman et al. 2008). Also, modulation of genes involved in focal adhesion and actin cytoskeleton rearrangement, both of importance in tissue invasion and metastasis (Hanahan and Weinberg 2000) is observed. Although these pathways are not statistically enriched in a DAVID analysis, they are correlated to the modulated genes using EASE, providing mechanistic insights on how US28 may induce transformation and tumor development. Another analysis of the microarray data, focusing on cancer-related genes and NF-κB transcriptional targets reveals modulation of genes involved in several forms of cancer that have previously been linked to HCMV infection such as colorectal and breast cancers (Ptgs2/Cox-2, Nr4a2, Serpine1, Csf1 and Gadd45b). Genes participating in multiple forms of cancer (e.g. Net1, Cttn, Runx1, Mdm2, Timp3 and Hgf) are also modulated by US28 expression in NIH-3T3 cells. Although a more systematic approach is required to obtain insight in the oncogenic signaling networks activated by US28, this study points towards several oncogenic targets that are modulated by US28.

Since US28 constitutively activates NF-κB signaling, it is not surprising to observe modulation of NF-κB target genes in US28-expressing cells (Figure 1D). One of such genes under control of NF-κB is COX-2 (Smith, DeWitt et al. 2000), which appears to be highly upregulated in US28-expressing cells and tumors (Table 1). COX-2 is of specific interest since it is a key mediator of inflammation and it is now well established that it contributes to the pathogenesis of several forms of cancer. This enzyme is commonly expressed in both premalignant lesions and malignant

tumors of e.g. colon, lung, head, neck and breast (Turini and DuBois 2002). In view of the role of COX-2 in tumor development and its upregulation in herpesvirus-infected cells, this enzyme has been suggested to participate in neoplasia induced by some of the oncogenic herpesviruses, including KSHV and EBV (Shelby, Nelson et al. 2005). Of interest, HCMV possesses oncomodulatory properties (Cinatl, Vogel et al. 2004) and also upregulates COX-2 expression within a few hours after infection (Zhu, Cong et al. 2002).

Since our microarray studies reveal marked increases in COX-2 expression in US28-expressing cells, and US28 was shown earlier to upregulate expression of VEGF and cyclin D1 (Maussang, Verzijl et al. 2006), both known to be regulated by COX-2 (Dorsam and Gutkind 2007), COX-2 is a likely determinant in the US28-induced tumor formation. Our studies show that US28 expression is associated with upregulation of COX-2 at both the mRNA and protein levels via activation of NF-κB (Figure 2 and 3). As expected, NF-κB is also implicated in the US28-induced VEGF promoter activity (Figure 3D). The G-protein uncoupled mutant US28-$R^{129}$A does not activate NF-κB nor upregulate COX-2 expression (Figure 2 and 3), indicating a role for G proteins in the US28-mediated COX-2 upregulation (Casarosa, Bakker et al. 2001).

In order to further demonstrate the contribution of COX-2 in the US28-induced tumor formation, we performed *in vitro* assays and intervention studies in our US28 tumor xenograft model using the NSAID COX-2 inhibitor Celecoxib (Figure 5). Several clinical studies have provided encouraging evidence of preventive effects of NSAIDs in cancers of colon, lung, breast and prostate (Pereg and Lishner 2005). Although some of these drugs were associated with cardiovascular toxicity, recent studies presented Celecoxib as one of the coxibs possessing least side effects (Howes 2007). Various animal studies reported effective inhibition of cell growth *in vitro* and tumor growth *in vivo* upon Celecoxib treatment (Cui, Yu et al. 2005; Zhang, Chen et al. 2005; Ragel, Jensen et al. 2007). *In vivo*, Celecoxib treatment of mice injected with US28-expressing cells severely delays and impairs tumor formation. After 3 weeks, no tumors are formed in the Celecoxib-treated mice, while tumors are apparent in the control group, indicating that COX-2 expression is of importance in early tumorigenic events induced by US28. But in the long term, Celecoxib-treated mice also present tumors, indicating that COX-2 is not the sole factor responsible for US28-induced tumor formation. Importantly, Celecoxib treatment reduces the growing rate of the tumor compared to untreated mice ($p<0.001$ in Logrank test), confirming the involvement of COX-2 in tumor development. In addition, Celecoxib impairs the angiogenic phenotype induced by US28,

both *in vitro* by significantly ($p<0.0001$) decreasing the production of VEGF in US28-expressing cells, and *in vivo*, by reducing angiogenic activity in US28-derived xenografts. As such, the impairment of the COX-2/VEGF axis by Celecoxib could be accounted for the decreased angiogenesis and subsequent slower development of the tumors *in vivo*. It is important to note that our microarray analysis indicates that other oncogenes beside COX-2 are likely to be implicated in US28-induced tumor progression. The observed inhibitory effect of Celecoxib on tumor formation are most likely attributed to the functional inhibition of COX-2, although some COX-2 independent effects by Celecoxib have been reported (Grosch, Maier et al. 2006). Other targets of Celecoxib include e.g. Cyclin D1 (Patel, Subbaramaiah et al. 2005) and NF-κB (Subhashini, Mahipal et al. 2005), both upregulated in US28-transfected cells. Thus, impairment of US28-induced tumorigenesis by Celecoxib, either through direct or indirect COX-2 inhibition, involves inhibition of proliferative signaling proteins that are constitutively activated by US28.

Cells infected with the AD169 strain devoid of US28 (AD169-ΔUS28) show a reduced activation of NF-κB and upregulation of COX-2 compared to cells infected with the WT virus (Figure 4). *In vivo*, US28 expression has been shown in lung transplant immunosuppressed patients presenting HCMV primary infection or reactivation (Boomker, Verschuuren et al. 2006). Because of the US28-mediated increases in NF-κB activation and COX-2 upregulation in infected cells *in vitro*, US28 may be implicated in virus-associated pathologies by further enhancing and/or contributing to increases in COX-2 expression.

We have previously shown that US28 constitutive activity is mediated by $G\alpha_q$ and $G\beta\gamma$ proteins (Casarosa, Bakker et al. 2001; Maussang, Verzijl et al. 2006). The constitutive activation of COX-2 or VEGF promoter by US28 could not be modulated by any of the CC and CX3C chemokines (CCL2, CCL5 and CX3CL1) known to bind this viral GPCR (data not shown). Ligand-stimulation of US28 has however been shown to promote cell migration via activation of $G\alpha_{12/13}$ proteins (Melnychuk, Streblow et al. 2004). As such, the $G\alpha_q$ pathway may be preferentially activated constitutively by US28 during oncogenic transformation, while the ligand-induced signaling may be favorable for the migration of US28-expressing cells. We therefore postulate that upon HCMV infection, US28 is expressed and constitutively activates NF-κB in a ligand-independent manner. US28 potentially activates $G\alpha_q$ and $G\beta\gamma$ (Casarosa, Bakker et al. 2001) to induce the expression of inflammatory proteins such as COX-2 (Smith, DeWitt et al. 2000). This enzyme is responsible for the synthesis of PGE2, which through activation of its cognate receptors EP1-4 leads to a subsequent enhancement of cell proliferation (Cyclin D1) and promotion of angiogenesis (VEGF) (Dorsam

and Gutkind 2007). US28-dependent increases of at least COX-2 early after infection might be sufficient to catalyze inflammatory processes, which may contribute to or enhance tumor formation (Figure 5D). Hence, the development of HCMV-related proliferative diseases might in part be ascribed to the ability of US28 to modulate expression of COX-2.

## Materials and Methods

**Cell culture.** The mock (empty pcDEF3 expression vector) and US28 stably transfected NIH-3T3 cell lines were cultured as previously described (Maussang, Verzijl et al. 2006). The human embryonic kidney cell line HEK 293T was cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and transfected using polyethylenimine (PEI). The human foreskin fibroblast BJ cell line was maintained in Minimum Essential Medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% fetal bovine serum.

**Chemokine binding and inositol phosphate accumulation experiments.** Stably transfected NIH-3T3 cells and HCMV-infected BJ cells were analyzed for radiolabelled chemokine binding and inositol phosphate formation as previously described (Maussang, Verzijl et al. 2006).

**Microarray analysis.** NIH-3T3 cells stably transfected with either mock or US28 were serum starved overnight with 0.5% calf serum containing medium. Total RNA was isolated from two independent clones for both cell lines with the RNeasy kit (QIAgen) followed by cDNA synthesis and overnight biotin-labeled cRNA amplification (MessageAmp II aRNA Amplification, Ambion). 20 μg of biotin-labelled cRNA was hybridized to Affymetrix Mouse Genome 430 2.0 arrays and processed according to Affymetrix procedures. Arrays were normalized (RMA) and analyzed using LIMMA statistical package (Smyth 2005). The data have been deposited in NCBI's Gene Expression Omnibus (Edgar, Domrachev et al. 2002) and are accessible through GEO Series accession number GSE13567 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13567). Genes differentially expressed between mock and US28-transfected cells were selected based on stringent false discovery rates (FDR $\leq$ 0.02). The retrieved gene lists were analyzed with DAVID and EASE softwares (Dennis, Sherman et al. 2003; Hosack, Dennis et al. 2003) and compared to lists of cancer-related genes (http://cbio.mskcc.org/CancerGenes) and to NF-κB transcriptional targets (adapted from http://jura.wi.mit.edu/young_public/nfkb/literature_targets.xls).

**Quantitative real time-PCR (qPCR).** For biological validation of microarray data by qPCR analysis, total RNA was isolated from NIH-3T3 cells and from US28 xenografts using the RNeasy kit (Qiagen) and reverse transcribed using iScript cDNA synthesis kit (BioRad), according to the manufacturers' instructions. qPCR primers (from Invitrogen or Isogen) used are described in Table 3. PCR reactions were performed using SYBR Green mix with MyiQ Real-Time PCR detection system (Bio-Rad). qPCR on HCMV-infected cells was performed with the ABI-Prism 7700 instrument and the SYBR Green PCR Master Mix (Applied Biosystems). Data were evaluated with the sequence detection software (SDS) version 1.9.1 (Applied Biosystems) and the second derivative maximum algorithm. In addition to melting curve analysis, specificity of the PCR products were confirmed by running controls on agarose gel electrophoresis and subsequent DNA sequence analysis.

**Luciferase reporter gene experiments.** The NF-κB and VEGF promoter luciferase reporter genes were previously described (Casarosa, Bakker et al. 2001; Maussang, Verzijl et al. 2006) and the WT and mutated COX-2 promoter luciferase reporter gene plasmids were obtained from Dr Fresno (Iniguez, Martinez-Martinez et al. 2000). Luciferase activity was measured 24 h post transfection (RLU, relative light units).

**Viral experiments.** BJ cells were infected with the different HCMV virus strains (AD169-WT and AD169-ΔUS28) (Casarosa, Menge et al. 2003) with a M.O.I. of 3 and the different assays were performed 8 h post-infection. NF-κB activation was measured by transfecting cells with the NF-κB luciferase reporter gene 24 h before infection.

**Western Blot Analysis.** Quantification of COX-2 protein level was performed by Western blot on total cell lysates with a rabbit polyclonal COX-2 antibody (Cell Signaling Technology) and a mouse monoclonal β-actin antibody (Sigma).

**In vivo experiment.** All animal experiments were conducted according to the National Institutes of Health principles of laboratory animal care and Dutch national law [''Wet op de Dierproeven'' (Stb 1985, 336)], approved by the Dierexperimentencommissie from the VU Medical Center and performed in compliance with the protocol FaCh 05-02. US28 stably transfected NIH-3T3 cells (2 x 106) were injected s.c. into the flanks of 8 to 10-week-old female nude mice (Hsd, athymic nu/nu, 25–32 g, Harlan Laboratories Cambridge Research Biochemicals). The control group was fed with regular mouse chow (Teklad Harlan), while the Celecoxib group was fed 1 day prior to the cell injection with mouse chow containing 1500 ppm Celecoxib (Pfizer) ad libitum.

**Immunohistochemistry.** Cryosections of US28-induced xenografts were stained for the presence of CD31 using a rat anti-mouse CD31 antibody (BD PharMingen) with an HRP-conjugated mouse anti-rat antibody (Zymed Laboratories).

**Table 3.** Sequences of the primers used for quantitative RT-PCR experiments.

| Gene symbol | Species | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|---|
| Gapdh | Mus Musculus | TCAACGGCACAGTCAAGG | ACTCCACGACATACTCAGC |
| Mef2c | Mus Musculus | GGAACACGCCTGTCACCTAAC | TGAATGAGTGCCATACGCCAATG |
| Cox-2 | Mus Musculus | CCTCTGCGATGCTCTTCC | TCACACTTATACTGGTCAAATCC |
| Tgfb2 | Mus Musculus | CTCCACAGTGTTTCAGCCTTTTC | GAACCATCCATCCCAGAAGCC |
| Cxcl12 | Mus Musculus | GGCTCCTTTATCCAGTTCAGTGC | ACAGAGGTGAGAAGCGGAAGTC |
| US28 | HCMV | TGACCGACTACGACTACTTAGAGG | CTGAGCGGGATCACGAAAGC |
| β-ACTIN | Homo Sapiens | CGGGACCTGACTGACTACCTC | CTCCTTAATGTCACGCACGATTTC |
| COX-2 | Homo Sapiens | GGAGCACCATTCTCCTTGAA | GAAAACCCACTTCTCCACCA |
| U1A | Homo Sapiens | GCAGCTTATGCCAGGACAGAT | TTGGTGAGGAACAAGATGTGATTC |
| RPS11 | Homo Sapiens | AAGCAGCCGACCATCTTTCA | CGGGAGCTTCTCCTTGCC |

# V

# HCMV-encoded chemokine receptor US28 mediates proliferative signaling by establishing a positive feedback loop via activation of the IL-6/STAT3 axis

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Adapted from Slinger, Maussang et al., *Manuscript in preparation*.

**Abstract**

US28 is a viral G protein-coupled receptor encoded by the human cytomegalovirus. Besides its ability to bind and internalize chemokines, we have previously shown that US28 constitutively activates signaling pathways linked to proliferation. In this study, we show enhanced levels of vascular endothelial growth factor (VEGF) and interleukin (IL)-6 in the supernatant of US28 expressing NIH-3T3 cells. The rise in IL-6 appears to be associated with increased activation of the signal transducer and activator of transcription 3 (STAT3), via the upstream activation of Janus activated kinase JAK1. Using conditioned growth medium, IL-6 neutralizing antibodies, an inhibitor of the IL-6 receptor, and shRNA targeting IL-6, we show that US28 activates the IL-6/JAK1/STAT3 signaling axis through a positive feedback loop. This loop is initiated in an US28-dependent manner through activation of the NF-κB transcription factor. Treatment of cells with either an inhibitor of STAT3 (JSI-124) or JAK1 (Pyridone 6) inhibit US28-mediated [$^3$H]-thymidine incorporation, further implying a key role for STAT3 in the US28-induced proliferative phenotype. Taken together, US28 appears to activate proliferative signaling via a novel signaling pathway, by establishing a positive feedback loop via activation of the IL-6/STAT3 axis.

## Introduction

Human cytomegalovirus (HCMV), a member of the family of β-herpesviruses, is widely present amongst the population establishing latent infection in up to 90% of the individuals (Gandhi and Khanna 2004). While infection with HCMV in immunocompetent individuals is asymptomatic, it may cause pathologies such as pneumonitis, hepatitis and retinitis in immunocompromised hosts (Gandhi and Khanna 2004). Furthermore, HCMV infection has been proposed to promote the development of colon cancer (Stassen, Vega-Cordova et al. 2006) and malignant glioma (Harkins, Volk et al. 2002).

The presence of four G-protein coupled receptors (GPCR) in the HCMV genome, US28, US27, UL33, and UL78, homologous to human chemokine receptors (Chee, Satchwell et al. 1990; Gompels and Macaulay 1995) is intriguing. Chemokine receptors belong to the class of GPCRs and are involved in regulation of the immune system (Thelen and Stein 2008), but also in various aspects of oncogenesis (Balkwill 2004). The HCMV-encoded GPCR US28, presenting highest homology to the human chemokine receptors CCR5 and CX3CR1, has been studied most extensively (Vischer, Leurs et al. 2006). US28 binds a wide variety of chemokines, including: CCL2, CCL5, and CX3CL1 (Bodaghi, Jones et al. 1998). Therefore, presence of US28 may cause suppression of the hosts's immune response against HCMV infection by sequestering chemokines (Randolph-Habecker, Rahill et al. 2002). In addition, US28 possesses constitutively active signaling properties and shows G-protein promiscuity which enables it to hijack the host cell signaling machinery (Casarosa, Bakker et al. 2001).

Similarly to chemokine receptors, viral GPCRs also play a role in oncogenesis and tumor growth. Transgenic mice expressing the Kaposi's sarcoma associated herpesvirus (KSHV)-encoded chemokine receptor ORF74 developed lesions resembling those induced by its virus (Yang, Chen et al. 2000). Likewise, US28 was shown to induce various oncogenic responses *in vitro* (increased cyclin D1 expression, vascular endothelial growth factor (VEGF) production, and cyclooxygenase-2 (COX-2) upregulation) when stably expressed in NIH-3T3 fibroblasts. Furthermore, in HCMV-infected cells US28 appears to contribute to increases of HCMV-induced VEGF promoter activity and COX-2 expression (Maussang, Verzijl et al. 2006; Maussang, Langemeijer et al. 2009). US28 is also capable of promoting tumor formation in a mouse xenograft model (Maussang, Verzijl et al. 2006).

To further investigate the molecular mechanism by which US28 contributes to the progression of oncogenesis, we analyzed US28-induced release of cytokines. An antibody array, recognizing different

chemokines, growth factors, and cytokines was used to identify factors secreted by US28-expressing NIH-3T3 cells. Interestingly, US28 appears to upregulate the expression of interleuking (IL)-6, reported to be induced upon HCMV infection (Rahbar, Bostrom et al. 2003; Halwachs-Baumann, Weihrauch et al. 2006), and implicated in oncogenesis (Wei, Kuo et al. 2001; Yu, Wang et al. 2002). Subsequently, we identified a key role for the IL-6/STAT3 axis in US28-mediated proliferative signaling. These data reveal a positive feedback loop initiated by US28 that is crucial for the proliferative phenotype displayed by US28-expressing cells.

## Results

### IL-6 and VEGF secretion are increased in US28 expressing cells

Previously, we have shown that NIH-3T3 cells stably expressing US28 display oncogenic properties (Maussang, Verzijl et al. 2006; Maussang, Langemeijer et al. 2009). Injection of US28 transformed NIH-3T3 cells into nude mice resulted in formation of tumors (Maussang, Verzijl et al. 2006). As angiogenic factors are required for the formation of large tumors (Hanahan and Weinberg 2000), secretion of these factors by US28 expressing NIH-3T3 cells was studied. Conditioned medium from US28 expressing NIH-3T3 cells was analyzed and compared to mock-transfected NIH-3T3 using a mouse angiogenesis antibody array. The antibody array analysis showed a marked increase of both IL-6 and VEGF (Figure 1), which, for the latter, was in accordance with earlier observations (Maussang, Verzijl et al. 2006). Furthermore, decreased levels of CCL2, a chemokines known to bind to US28 (Bodaghi, Jones et al. 1998), were observed. In contrast, the levels of CCL11 and CXCL4, chemokines that do not bind US28, remained unaffected. As IL-6 has been reported to be an important factor in oncogenesis (Hanahan and Weinberg 2000; Kuilman, Michaloglou et al. 2008), we further investigated the importance of IL-6 in US28-induced proliferative signaling.

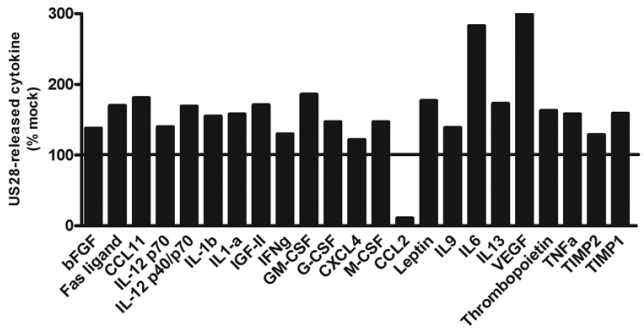


**Figure 1.** Increased IL-6 and VEGF secretion in US28 transfected cells. Medium from NIH-3T3 cells stably transfected with US28 was compared with medium obtained from mock transfected cells. Excreted cytokines and growth factors were analyzed using an antibody array. A representative result of three separate experiments is shown as a percentage of protein levels in US28 expressing cells compared to mock transfected cells.

## STAT3 phosphorylation and STAT3-driven transcriptional activation is induced by US28

Since IL-6 is known to activate STAT3 signaling through binding to its cognate receptor IL-6Rα (Heinrich, Behrmann et al. 1998), we assessed the phosphorylation status of STAT3. In accordance with the observed increase in IL-6 levels in the supernatant of US28-transfected cells, phospho-STAT3 levels were markedly increased in US28-expressing cells, compared to mock-transfected cells (Figure 2A). No STAT3 phosphorylation was observed in cells expressing the G-protein uncoupled US28-$R^{129}$A mutant (Waldhoer, Casarosa et al. 2003). Furthermore, a luciferase-based STAT3 reporter gene assay showed that STAT3-driven transcriptional activation was increased in HEK 293T transiently transfected with US28, but not mock- or US28-$R^{129}$A-transfected cells (Figure 2B). Expression of US28 in both HEK 293T (Figure 2C), and NIH-3T3 (Figure 2D) was confirmed using [$^{125}$I]-CCL5 binding.

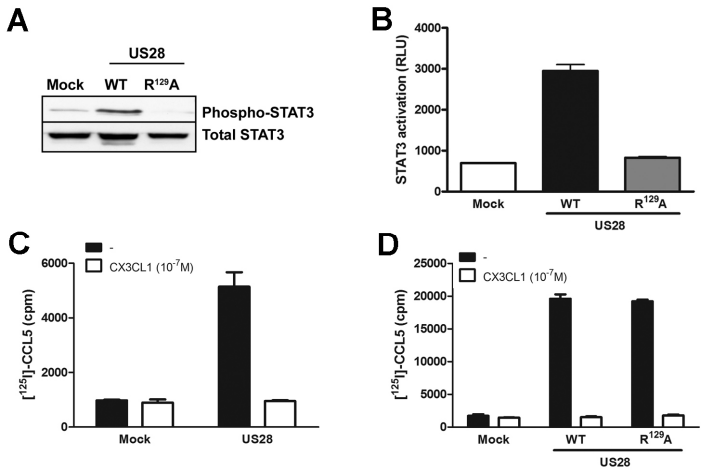


**Figure 2.** US28 induces STAT3 activation **A**, STAT3 phosphorylation levels are increased in NIH-3T3 stably transfected with US28 compared to mock cells. The G-protein uncoupled mutant US28-$R^{129}$A shows no increase in STAT3 phosphorylation. **B**, in HEK 293T cells, STAT3-driven transcriptional activation is only observed when the US28 wild-type receptor is expressed. **C**, [$^{125}$I]-CCL5 binding to US28, displacement with $10^{-7}$ M CX3CL1 in HEK 293T. (D) [$^{125}$I]-CCL5 binding to US28 and US28 $R^{129}$A, displacement with $10^{-7}$ M CX3CL1 in stably transfected NIH-3T3.

## US28-induced STAT3 signaling is mediated by JAK1 and NF-κB

To further examine US28-mediated STAT3 signaling, the possible role of upstream kinases involved in STAT3 phosphorylation (Murray 2007; Yu, Kortylewski et al. 2007), was investigated using specific inhibitors. These analyses were performed both in transiently transfected HEK 293T cells using STAT3 reporter gene assays and NIH-3T3 cells stably

expressing US28, in which STAT3 phosphorylation was determined using Western blot analysis. Overnight treatment of US28 expressing HEK 293T cells with 10 μM AG-490 (JAK2 inhibitor), Pyridone-6 (pan-JAK inhibitor), PP-2 (Src inhibitor), and Tyrene CR-4 (Abl inhibitor) showed different effects on STAT3-controlled reporter gene activation (Figure 3A). Treatment with Pyridone-6 (P6) resulted in the strongest inhibition of US28-induced STAT3 signaling (±50%), whereas the other kinase inhibitors did not affect STAT3 activation. As $G\alpha_o$ has been previously described to activate STAT3 (Ram, Horvath et al. 2000), we assessed a possible $G\alpha_o$ component in US28-induced STAT3 signaling by treating cells with 100 ng/ml pertussis toxin (PTX) (Figure 3A). No significant reduction in STAT3 reporter gene activity could be observed.

Since IL-6 production is known to be controlled by the transcription factor NF-κB (Libermann and Baltimore 1990; Son, Jeong et al. 2008) which has been shown to be constituvely activated via $G\alpha_q$ and the $G\beta\gamma$ subunits in cells expressing US28 (Casarosa, Bakker et al. 2001), we treated cells with NF-κB inhibitor BAY11-7082 (Pierce, Schoenleber et al. 1997; Lappas, Yee et al. 2005). This resulted in a marked decrease (approximately 80%) of STAT3 transcriptional activation (Figure 3A).

All kinase inhibitors, except PP-2, were also used to assay STAT3 phosphorylation in the US28 expressing NIH-3T3 cells. In this assay, cells were treated for 30 minutes with 10 μM of each inhibitor. A strong reduction of STAT3 phosphorylation was observed only after treatment with P6 (Figure 3B), indicating that US28-mediated STAT3 phosphorylation is exerted by the JAK1 kinase.

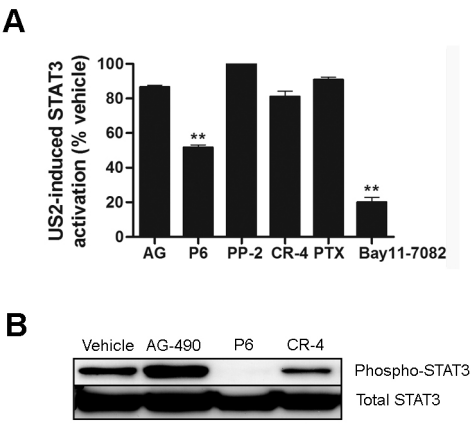


**Figure 3.** US28 activates STAT3 via JAK1. **A**, STAT3 driven transcriptional activation is inhibited by treatment with 10 μM of Pyridone 6 (P6, pan-JAK kinase inhibitor) (**p < 0.01 compared to vehicle treated), whereas similar concentrations AG-490 (AG, JAK-2 inhibitor), PP-2 (Src inhibitor), and Tyrene CR-4 (Abl inhibitor) have no or a minor effect. Also, 100 ng/ml pertussis toxin (PTX) has no effect on STAT3 activation. Treatment with the NF-κB inhibitor BAY11-7082 results in an approximately 80% reduction of STAT3 reporter gene activation (**p < 0.01 compared to vehicle treated). **B**, NIH-3T3 stably transfected with US28 were treated for 30 minutes with the different kinase inhibitors. Only treatment with 10 μM P6 shows inhibition of STAT3 phosphorylation.

## US28 activates the IL-6/STAT3 axis indirectly

To assess whether US28 directly or indirectly, via activation of NF-κB, induces increased IL-6 production, we investigated the importance of US28 induced IL-6 release in the activation of STAT3. When mock and US28 expressing NIH-3T3 cells are treated with 10 ng/ml IL-6 we observed a marked phosphorylation of STAT3 (Figure 4A).

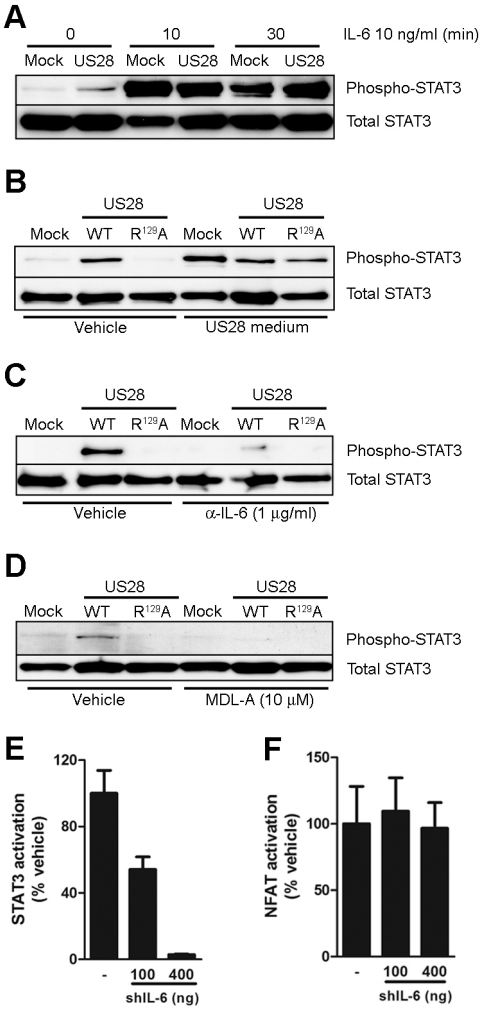


**Figure 4.** US28 induces a positive feedback loop which involves IL-6 signaling. **A**, incubation with 10 ng/ml IL-6 during 10 or 30 min results in STAT3 phosphorylation in both Mock and US28 transfected NIH-3T3. **B**, Conditioned medium obtained from NIH-3T3 transfected with US28 is able to induce STAT3 phosphorylation after a 90 minute incubation in both mock, US28 wild-type, and US28-$R^{129}A$ transfected NIH-3T3. **C**, Incubation with 1 μg/ml IL-6 neutralizing antibody for 90 minutes leads to decreased STAT3 phosphorylation. **D**, An overnight incubation with 10 μM of gp130 inhibitor Madindoline-A (MDL-A) also inhibits STAT3 phosphorylation in US28-expressing cells. **E**, HEK 293T cells expressing US28, were co-transfected with different amounts of shIL-6. At 100 ng shIL-6 / $10^6$ cells STAT3 activation is reduced by almost 50% (*$p < 0.05$ compared to vehicle transfection), whereas using 400 ng shIL-6 per $10^6$ cells completely abolishes STAT3 activation (***$p < 0.001$ compared to vehicle transfection). **F**, Co-transfection of shIL-6 is unable to significantly alter NFAT reporter gene activation.

Mock-, US28-wildtype and US28-$R^{129}A$-transfected cells were treated `both mock- and US28-$R^{129}A$-transfected cells, as assessed by Western

blot analysis (Figure 4B). Furthermore, a 90 min incubation of US28 expressing NIH-3T3 cells with 1 μg/ml IL-6 neutralizing antibody resulted in strongly attenuated STAT3 phosphorylation levels (Figure 4C).

To evaluate the mode of action of IL-6, we used Madindoline-A, which inhibits the formation of gp130 homodimers thus inhibiting signaling of IL-6Rα/gp130 complex (Hayashi, Rho et al. 2002). Treatment of US28-expressing NIH-3T3 cells with 10 μM Madindoline-A completely inhibited STAT3 phosphorylation (Figure 4D). To investigate the involvement of IL-6 in the US28-induced activation of STAT3 in HEK 293T, shIL-6, the shRNA targeting and downregulating IL-6 (Kuilman, Michaloglou et al. 2008) was expressed in conjunction with the STAT3 reporter gene. Co-transfection of shIL-6 resulted in a complete abolishment of US28-induced STAT3 transcriptional activity (Figure 4E). Co-transfecting the same shRNA with a NFAT reporter gene did not significantly alter the US28-induced NFAT constitutive signaling (Figure 4F). These results indicate that, both in HEK 293T and NIH-3T3 cells US28-induced IL-6 release mediates STAT3 activation, via its cellular receptor.

## STAT3 is involved in the US28-induced proliferative phenotype

To demonstrate the role of IL-6 driven STAT3 signaling in the US28-mediated proliferative phenotype, we analyzed the effects of inhibitors against STAT3, JAK1, and gp130 on US28-mediated proliferation. The specific STAT3 inhibitor JSI-124 (Blaskovich, Sun et al. 2003) inhibited US28 mediated STAT3 signaling in HEK 293T cells (Figure 5A) with an $IC_{50}$ of approximately 500 nM. Subsequently, we used a [$^3$H]-thymidine incorporation assay to determine the effect of various inhibitors of the IL-6/STAT3 pathway on the proliferation of mock- and US28-expressing NIH-3T3 cells. The STAT3 inhibitor JSI-124 and the JAK inhibitor P6 were tested in the same experimental setup. Treatment with either compound resulted in a strong reduction of proliferation in the US28-transfected cells (Figure 5B). Since US28 expression induces expression of VEGF, and because this angiogenic factor is in part regulated by STAT3 (28), we examined the role of the IL-6/STAT3 in the US28-induced VEGF promoter activation (14). Similarly to STAT3 activation, VEGF promoter activation was also impaired in the presence of STAT3 (JSI-124) and JAK (P6) inhibitors (Figure 5C). In addition, co-expression of shIL-6 resulted in a marked reduction of US28-induced activation of VEGF promoter activation (Figure 5C).

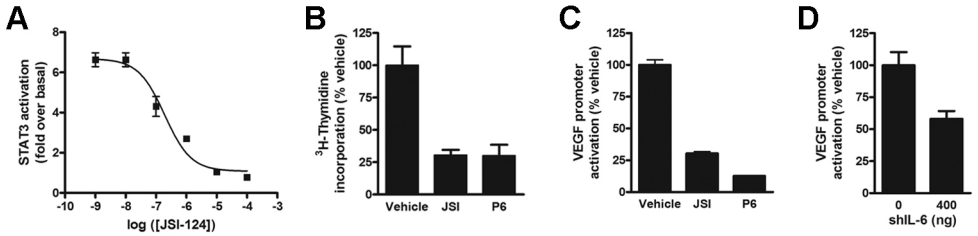


**Figure 5.** STAT3 plays a critical role in US28-induced proliferation **A**, The STAT3 inhibitor JSI-124 inhibits STAT3 response element activation in HEK 293T after a 24 hour incubation. **B**, over-night treatment with either JSI-124 (JSI, 500 nM) or P6 (500 nM) inhibits DNA synthesis in NIH-3T3 cells expressing US28. **C**, knock-down of IL-6 with 400 ng shIL-6 400 ng per $10^6$ in HEK 293T cells expressing US28 inhibits VEGF promoter activation. **D**, treatment of HEK 293T cells co-transfected with US28 and VEGF reporter gene with either 10 μM P6 or 300 nM JSI-124 inhibits VEGF promoter activation.

## Discussion

Several viral infections have recently been convincingly linked to the formation of tumors. Kaposi sarcoma-associated herpes virus (KSHV) and Human papilloma virus (HPV), for example, are considered the etiological agents of Kaposi sarcoma and cervical cancer, respectively (Ganem 1997; Munoz, Bosch et al. 2003). Although the human cytomegalovirus (HCMV) is not considered an oncogenic virus like KSHV and HPV (Damania 2004), HCMV has been suggested to act as an oncomodulator (Michaelis, Doerr et al. 2009). HCMV has been shown to interfere with several key cellular signaling pathways, leading to enhanced survival and angiogenesis, as well as alterations in cell motility and adhesion (reviewed in (Soderberg-Naucler 2006)). HCMV-encoded proteins are believed to drive these processes directly by activation of cellular signaling pathways and indirectly through induction of autocrine and paracrine signaling. One of the HCMV-encoded proteins, shown to induce a proliferative and angiogenic phenotype *in vitro* and *in vivo*, is the viral chemokine receptor US28 (Maussang, Verzijl et al. 2006). The molecular mechanism by which US28 causes this oncogenic behaviour is still subject of investigation.

In this study, we focused on US28-mediated secretion of soluble factors that may be involved in proliferative signaling. We analyzed a set of secreted growth factors, chemokines, and cytokines using an antibody array, and confirmed the previously reported increase in levels of VEGF in the growth medium of US28-expressing NIH-3T3 cells. Furthermore, CCL2 levels were found to be decreased, which may reflect sequestration of this chemokine by US28, as previously described (Bodaghi, Jones et al. 1998). Most interestingly, we found IL-6 levels to be elevated in the growth medium of US28-expressing cells.

IL-6 is a pro-inflammatory cytokine that induces rapid and strong phosphorylation of STAT3, via binding to its cognate receptor IL-6R$\alpha$ and subsequent activation of the tyrosine kinase subunit gp130 (Heinrich, Behrmann et al. 1998). Upon binding of the ligand to IL-6R, the gp130 subunits of the receptor complex activate JAK1, which subsequently results in activation of STAT3 and its target genes. Recent work by Kuilman *et al.* has shown that IL-6 is an important regulatory factor in melanoma and may play a pivotal role in the switch from senescence to oncogenesis (Kuilman, Michaloglou et al. 2008). STAT3 is a transcriptional regulator known to be up-regulated in solid tumors (Bromberg, Wrzeszczynska et al. 1999), as well as in lymphoma's (Mitchell and John 2005). Recent studies show that constitutive active gp130 mutants are responsible for increased STAT3 phosphorylation in hepatocellular tumors (Rebouissou, Amessou et al. 2009). As such,

STAT3 has been considered as a promising anti-cancer drug target (Buettner, Mora et al. 2002), which has stimulated the discovery of several inhibitors of STAT3 (Blaskovich, Sun et al. 2003; Bhasin, Cisek et al. 2008).

As IL-6 is known to be an important factor in oncogenesis via the IL-6/STAT3 axis (Wei, Kuo et al. 2001; Yu, Wang et al. 2002), we set out to investigate the role of IL-6 and STAT3 activation in US28-mediated proliferative signaling. To this end, we have used a combination of reporter gene assays, Western-blot analyses, and [$^3$H]-thymidine incorporation experiments to demonstrate that STAT3 plays a pivotal role in US28 induced proliferative signaling. Using an antibody recognizing phosphorylated STAT3, and the STAT3 reporter gene, we have shown that STAT3 is constitutively activated in cells expressing US28, whereas it is inactive in either mock transfected cells or cells expressing the G-protein uncoupled US28 mutant, US28-R$^{129}$A. A common factor in both US28 and IL-6 signaling is NF-κB, a transcription factor driving amongst others IL-6 expression (Libermann and Baltimore 1990; Shimizu, Mitomo et al. 1990; Son, Jeong et al. 2008). Using a specific NF-κB inhibitor, we show that NF-κB signaling is of key importance for the US28-mediated STAT3 activation. Since US28-induced NF-κB signaling is G-protein dependent (Casarosa, Bakker et al. 2001), this corroborates with the observed G-protein dependency of US28-mediated STAT3 activation.

Next, we investigated a potential positive feedback loop in which US28 triggers IL-6 production, which subsequently yields increased STAT3 activation via activation of the IL-6 receptor. In turn STAT3 may further induce IL-6 expression, thereby creating a positive feedback loop. Using conditioned medium from US28-expressing cells, we show that cells expressing US28 secrete factors that activate STAT3. Neutralizing antibodies raised against IL-6, chemical inhibitors of gp130, and shRNA directed against IL-6, further identify IL-6 as the key player in this positive feedback loop.

The importance of STAT3 signaling for the proliferative phenotype displayed by US28 expressing cells, was further confirmed by use of the STAT3 inhibitor JSI-124. In addition, inhibiting the upstream kinase JAK1 with the kinase inhibitor P6 also impairs US28-induced proliferation. Furthermore, constitutive STAT3 activity is known to upregulate VEGF expression and angiogenesis (Niu, Wright et al. 2002). Knock down of IL-6, with shIL-6, attenuates US28-induced VEGF promoter activity but not NFAT-mediated signaling, which is in line with the key roles we describe for IL-6 and STAT3 in US28-mediated proliferative signaling.

Based on these data we suggest the following model, displayed in Figure 6. Cells expressing US28 trigger production of IL-6, via activation of NF-κB. As a result, IL-6 binds to the gp130 subunits, inducing tyrosine phosphorylation of STAT3 via JAK1. The phospho-STAT3 protein dimerizes and translocates to the nucleus, to regulate STAT3 target genes. Amongst these target genes are proliferative and angiogenic factors such as Cyclin D1 (Sinibaldi, Wharton et al. 2000) and VEGF (Niu, Wright et al. 2002), respectively. As IL-6 has been shown to be a target gene of STAT3 (Sumimoto, Imabayashi et al. 2006), a positive feedback loop is created. Through experiments using conditioned medium we show that US28 is capable of initiating an autocrine feedback loop.

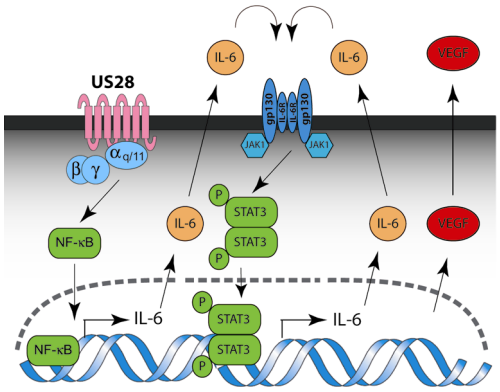


**Figure 6.** Model outlining the US28 positive feedback loop, which is started via the NF-κB pathway. One of the target genes of NF-κB is IL-6, which subsequently can activate the gp130 receptor that results in STAT3 phosphorylation and activation of its target genes (amongst others, VEGF). Since IL-6 is also a target gene of STAT3, STAT3 activation may further induce IL-6 release.

Our observations imply that cells infected with HCMV, through expression of US28, may induce secretion of IL-6 enhancing proliferative signaling in an autocrine manner; while inducing STAT3 activation in neighboring uninfected cells via paracrine effects. By locally altering cytokine levels US28 may facilitate tumor progression, thereby contributing to the oncomodulatory properties of HCMV.

Taken together, we show that the HCMV-encoded chemokine receptor mediates proliferative signaling via a novel signaling pathway, establishing a positive feedback loop via activation of the IL-6/STAT3 axis. Targeting the IL-6/STAT3 axis with inhibitors effectively inhibits US28-induced proliferative signaling. A systems approach, in which the kinetics of the interplay between NF-κB, IL-6, and the JAK/STAT axis are investigated, will be helpful to further delineate the role of IL-6, and STAT3 in US28-induced proliferative signaling.

## Materials and Methods

**Materials.** Pyridone 6 (pan-JAK inhibitor), Tyrene CR-4 (Abl inhibitor), AG-490 (JAK2 inhibitor), JSI-124 (STAT3 inhibitor), BAY117082 (NF-κB inhibitor) and PP-2 (Src inhibitor) were all purchased from Calbiochem (San Diego, CA, USA). Stocks were made in DMSO, except for JSI-124 which was dissolved in ethanol. The inhibitors were subsequently diluted in the culture medium. Madindoline-A was purchased from Alexis Biochemicals (Lausen, Switzerland). Tris base, pertussis toxin, and PEI were obtained from Sigma-Aldrich (St.Louis, MO, USA), other chemicals were obtained from Applichem (Darmstadt, Germany). Recombinant human CCL5, human CX3CL1, and murine IL-6 were obtained from Peprotech (Rocky Hill, NJ, USA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from PAA Laboratories (Pasching, Austria). Fetal bovine serum was purchased from Integro (Zaanstad, The Netherlands), and bovine serum was purchased from Invitrogen (Paisley, UK). [6-$^{3}$H]-Thymidine was obtained from GE Healthcare Life Sciences (Buckinghamshire, UK).

**Cell culture.** Human HEK 293T, and murine NIH-3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum or bovine serum, respectively. Transient transfections in HEK 293T were performed using the polyethylenimine method (Schlaeger and Christensen 1999). Stable clones of NIH-3T3 expressing US28 or US28-R$^{129}$A mutant (Maussang, Verzijl et al. 2006) were kept under a selective pressure of 400 μg/ml neomycin in the culture medium to ensure homogenous receptor expression the cells. Expression of US28 in both HEK 293T and NIH-3T3 was confirmed using [$^{125}$I]-CCL5 binding (specific binding was determined using CX3CL1 $10^{-7}$ M), as previously described (Casarosa, Bakker et al. 2001).

**Angiogenesis array.** An mouse angiogenesis array (Raybiotech, Norcross, GA, USA) was used according to manufacturers instructions to determine relative amounts of cytokines and chemokines involved in angiogenesis.

**Antibodies and Western-blot analysis.** For Western-blot analysis, monoclonal antibodies raised against phospho-STAT3 and total STAT3 were obtained from Cell Signaling (Boston, MA, USA). Neutralizing antibody against murine IL-6 was purchased from BD Biosciences (Franklin Lanes, NJ, USA). A Biorad (Hercules, CA, USA) minigel system was used to perform SDS/PAGE, and a Biorad electroblot system was used to transfer protein samples on a 0.45 μm nitrocellulose membrane. Cells were lysed in RIPA-buffer with protease inhibitor cocktail (α-complete, Roche), 1 mM PMSF, 1 mM $NaVO_4$, and 1 mM NaF added. Samples were normalized using the BCA total protein determination kit obtained from Thermo Fisher Scientific (Rockford, IL, USA).

**Reporter gene analysis.** The Ly6E STAT3-response luciferase construct (Ram, Horvath et al. 2000) was used for STAT3 activation measurements. To determine STAT3 activation, $10^{6}$ HEK 293T cells were transfected with 625 ng Ly6E STAT3-response luciferase construct, with or without pcDEF3 containing a gene encoding HA-US28. When inhibitors were used, they were added immediately following transfection. Total DNA amounts were kept constant by adding empty vector. Luciferase activity was measured 24 hours after transfection using a Victor$^{2}$ multilabel platereader from Perkin-Elmer (Waltham, MA, USA).

The NFAT reporter gene, pNFAT-luc was purchased from Stratagene (La Jolla, CA, USA). To measure VEGF promoter activation, the pGL2-VEGF-Luciferase construct, kindly provided by Dr. G. Pages (Institute of Signalling Development Biology and Cancer, Nice, France).

**IL-6 knock-down.** IL-6 knock-down experiments were performed with the pSuper.Retro.puro-sh-IL-6 construct kindly provided by Dr. D. Peeper (NKI, Amsterdam, the Netherlands) (Kuilman, Michaloglou et al. 2008). For the knock-down, HEK 293T cells were transiently transfected with shIL-6 and a STAT3, VEGF, or NFAT reporter gene. Luminescence measurements were taken 48 hours post-transfection.

**[$^3$H]-Thymidine incorporation**. Cell proliferation in NIH-3T3 cells was measured using [$^3$H]-thymidine incorporation. Cells were serum-starved overnight before labeling in DMEM supplemented with 0.5% bovine serum containing 1 μCi/ml $^3$H-Thymidine together with test compounds.

**Statistical analysis.** All experiments were performed at least three times in triplicate. When comparisons between treated and vehicle treated cells were made, Student T-test was performed using the GraphPad Prism software (San Diego, CA). Bars and error bars represent the mean and SEM, respectively.

# VI

# The Epstein Barr virus-encoded G protein-coupled receptor BILF1 induces B cell apoptosis *in vivo*

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Adapted from Maussang, Furtado et al., *Manuscript in preparation*.

**Abstract**

The gamma-herpesviruses, Kaposi's sarcoma associated herpesvirus (KSHV) and Epstein-Barr virus (EBV), possess transforming abilities and are responsible for the development of proliferative diseases. These viruses encode viral G protein-coupled receptors (vGPCRs) that are expressed during lytic phases of infection and exhibit constitutive signaling properties. While the KSHV-encoded vGPCR ORF74 has previously been linked to cellular proliferation and directly implicated in the development of KSHV-related diseases, little information is available on the function of the EBV-encoded vGPCR BILF1. We investigated whether, similarly to ORF74, BILF1 possesses proliferative properties. Since EBV presents a tropism towards B cells, transgenic (TG) mice expressing BILF1 within this population of lymphocytes were generated. TG animals expressing BILF1 exhibited a surprising lymphopenia that did not result from abnormal development of B cells, but from increased apoptosis of B cells. The depletion of peripheral B cells led to a marked decrease in the circulating levels of IgA and IgG subclasses, but not IgM that mediate immune responses in the early stage. In addition, BILF1 transgenic mice were compromised in their ability to respond to T cell-dependent antigens. Taken together, these data indicate that the EBV-encoded lytic gene BILF1 possesses pro-apoptotic properties and, unlike the KSHV-encoded ORF74, this vGPCR is likely not to be involved in viral proliferative conditions.

## Introduction

Herpesviruses are ubiquitous entities involved in various pathological conditions. In particular, gamma-herpesviruses are known to possess transforming properties and to be involved in the development of proliferative diseases. For instance, the γ1-human herpesvirus KSHV is the etiological agent of Kaposi's sarcoma, a common skin malignancy in immunosuppressed patients (Ablashi, Chatlynne et al. 2002). Also, the γ2-human herpesvirus EBV immortalizes lymphocytes *in vitro* and is the causative agent of lymphoproliferative disorders, such as Burkitt's and Hodgkin's lymphomas (Young and Rickinson 2004). Interestingly, herpesviruses encode for viral G protein-coupled receptors (vGPCRs) that present homology to human chemokine receptors, which play a role in immune regulation and the development of tumors (Mackay 2001; Balkwill 2004). Unlike their human counterparts, vGPCRs can signal in a ligand-independent, i.e. constitutive, manner (Rosenkilde, Smit et al. 2008). Upon viral infection, these vGPCR are expressed on the cell surface and activate a broad range of intracellular signaling pathways, which alter cellular functions and contribute to virus-associated pathogenesis (Rosenkilde, Smit et al. 2008). KSHV and EBV each encode a single vGPCR, respectively ORF74 and BILF1, whose expression occurs during the lytic phase of infection (Kirshner, Staskus et al. 1999; Beisser, Verzijl et al. 2005). Both vGPCRs have been shown to constitutively modulate gene transcription (Smit, Verzijl et al. 2002; Beisser, Verzijl et al. 2005).

ORF74 has been extensively studied with different *in vitro* and *in vivo* models. In vitro, this vGPCR induces the expression of inflammatory molecules and pro-angiogenic factors such as the vascular endothelial growth factor (VEGF) and constitutively induces cellular signaling in both autocrine and paracrine manners (Pati, Cavrois et al. 2001; Bais, Van Geelen et al. 2003; Sodhi, Chaisuparat et al. 2006). In addition, transgenic (TG) mice models confirmed that the lytic gene ORF74 uses autocrine and paracrine mechanisms to induce the formation of tumors that resemble human Kaposi's sarcoma lesions (Yang, Chen et al. 2000; Grisotto, Garin et al. 2006). As such, the lytic gene ORF74 mediates cellular transformation and viral pathogenesis. In contrast, little information is available on the EBV-encoded vGPCR BILF1. This receptor presents an early or immediate-early gene expression pattern during the lytic phase of infection (Beisser, Verzijl et al. 2005; Kutok and Wang 2006; Kaptein, Jungscheleger-Russell et al. 2008). To date, no ligand has been discovered for BILF1, which is thus still considered an orphan receptor. BILF1 constitutively activates several signaling pathways via

$G\alpha_{i/o}$ proteins and modulates both CREB- and NF-κB-mediated gene activation (Beisser, Verzijl et al. 2005). The important pathogenic function of the vGPCR ORF74 in proliferation and in the development of KSHV-related diseases led us to determine if BILF1 is also implicated in neoplastic pathologies. Since EBV presents a B cell tropism and induces the development of several B cell lymphoproliferative disorders (Kutok and Wang 2006), we targeted the expression of BILF1 in the B cell compartment of TG mice. TG animals expressing BILF1 developed normally but presented surprisingly low B cell numbers (B cell lymphopenia) in the blood and in the peripheral lymphoid organs. This phenotype was associated with increased apoptotic activity in B cells and subsequent lower immunoglobulin titers. Together, the results indicate that, unlike ORF74, BILF1 displays pro-apoptotic activity in vivo, leading to the destruction of B cells.

## Results

### Generation of BILF1 transgenic mice

EBV is a B cell-tropic virus, and BILF1 mRNA has been detected in several EBV positive B cell lines (Kaptein, Jungscheleger-Russell et al. 2008). To target expression to B cells, we expressed BILF1 with a transgene driven by immunoglobulin kappa light chain gene regulatory elements. To facilitate the detection of the BILF protein, we added sequences encoding the FLAG epitope to the BILF1 gene (Figure 1A). Thirteen founders were generated from the injection of this transgene into mouse eggs, of which four healthy animals showed BILF1 gene integration during genotyping analysis with standard PCR techniques (data not shown).

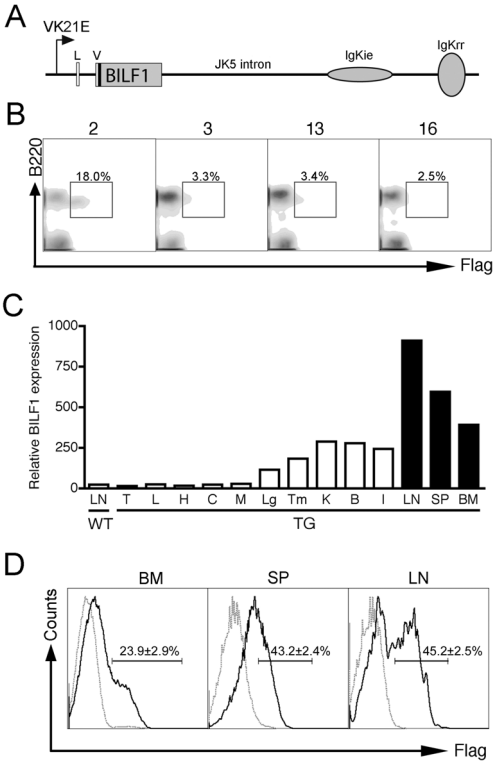


**Figure 1.** Schematic representation of BILF1 transgene and expression pattern of Flag-BILF1. **A**, Flag-tagged BILF1 gene is subcloned into an Igκ expression vector that includes the 5′ portion of the Vk21E gene (with leader (L) and V gene exons, (Robbiani, Colon et al. 2005)), the 3′UTR of the mouse kappa light chain gene, the kappa intronic sequence with enhancer (IgKie) and the kappa 3′ regulatory region (IgKrr). **B**, FACS analysis of non-permeabilized peripheral blood cells represent the repartition of $Flag^+$ and $B220^+$ cells in Igk-BILF1 transgenic founders. The numbers were used to identify the founders and show that founder 2 possesses the highest BILF1 cell surface expression in $B220^+$ cells. **C**, qPCR analysis of different organs of TG mice derived from founder 2 shows a high expression of BILF1 mRNA in the hematopoietic compartments. LN = lymph nodes, T = thyroid, L = liver, H = heart, C = colon, M = muscle, Lg = lung, Tm = thymus, K = kidney, B = brain, I = ileum, SP = spleen, BM = bone marrow. **D**, $CD19^+$ cells from bone marrow, spleen and lymph nodes of WT (grey dotted line) and TG (black line) mice derived from founder 2 show different levels of Flag-BILF1 expression on their cell surface. BM = bone marrow, SP = spleen, LN = lymph node.

To determine Flag-BILF1 protein expression in the transgenic founders, peripheral blood cells were stained with anti-B220 and anti-CD3, cell surface markers for B cells and T cells respectively, and anti-Flag antibodies. Fluorescent-activated cell sorting (FACS) analysis of the four healthy founders indicated that $Flag^+$ cells were restricted to $B220^+$ B cells (Figure 1B). $CD3^+$ T cells from either wild-type (WT) or TG mice showed no expression of Flag-BILF1 protein, highlighting the specific expression of BILF1 in B lymphocytes. The transgenic founder 2 had the highest expression of BILF1 and was used to generate the TG line referred to as Igκ-BILF1. TG mice derived from founder 2 were used to monitor BILF1 expression in several organs using quantitative PCR. As shown on Figure 1C, expression of BILF1 mRNA was mainly detected in the hematopoietic organs including bone marrow, spleen, and lymph nodes. BILF1 mRNA was also detected at lower levels in organs such as lung, thymus, intestine and kidney where low amounts of B cells are present. The Igκ-BILF1 line of TG mice used for this study showed that 23.9 ± 2.9 % of bone marrow B cells express Flag-BILF1 on their cell surface, while the spleen and lymph node B cells presented somewhat higher cell surface expression (respectively 43.2 ± 2.4 % and 45.2 ± 2.5 %) (Figure 1D).

## BILF1 expression induces B cell lymphopenia in the periphery

During initial phenotyping of the BILF1 transgenic founders, it appeared that the relative proportions of B cells in the blood were decreased in the TG mice compared to the WT littermates (Figure 2). TG founder 2 with the highest BILF1 expression presented the most pronounced B cell lymphopenia. Blood from founder mice expressing lower levels of BILF1 exhibited a milder lymphopenic phenotype that founder 2. Thus, increased BILF1 expression led to a more severe lymphopenia.

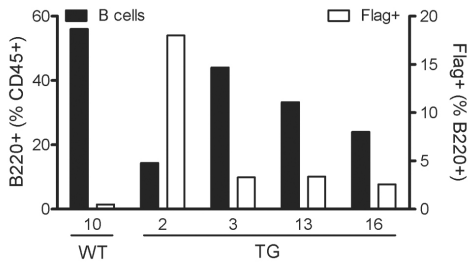


**Figure 2.** BILF1 expression correlates with B cell lymphopenia in the blood. Analysis of the blood from transgenic Igκ-BILF1 founders for the presence of B cells ($B220^+$) among $CD45^+$ cells, and BILF1-expressing cells ($Flag^+$) among $B220^+$ cells.

To confirm this observation, we analyzed progeny WT and TG Igκ-BILF1 mice from founder 2 at 8 to 10 weeks of age. FACS analysis of peripheral blood showed a significant decrease in the proportion of B cells in TG mice compared to their control littermates, suggesting that BILF1 expression caused B cell lymphopenia in the periphery (Figure 3A). Similar to peripheral blood, the relative and absolute number of B cells (CD19+ CD3- cells) was also greatly reduced in lymph nodes of TG mice (Figure 3B and C), whereas the absolute number of T cells was not affected (data not shown).

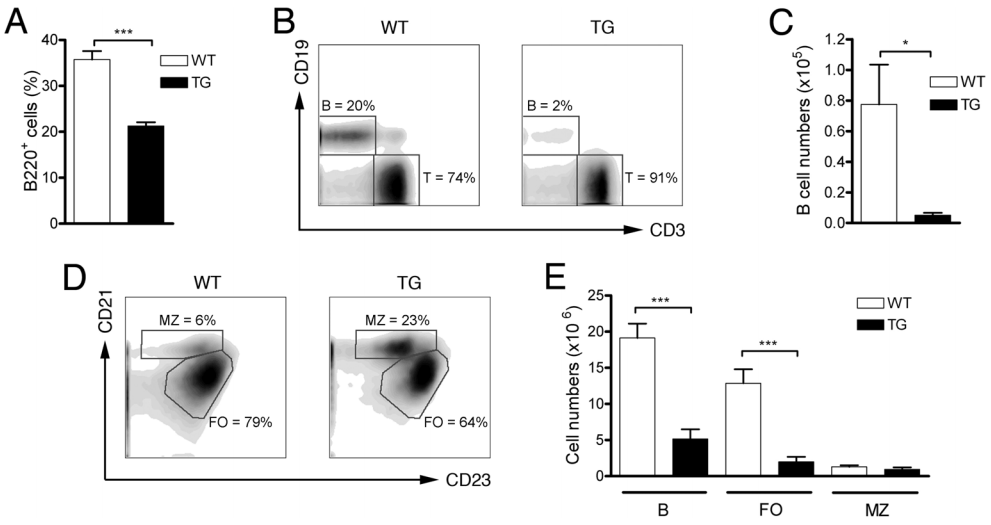


**Figure 3.** BILF1 expression induces a peripheral B cell lymphopenia in transgenic mice. **A**, Peripheral blood of Igκ-BILF1 TG mice presents a relative lower presence of $B220^+$ B cells (n = 18 WT vs 18 TG). **B**, FACS analysis of inguinal lymph nodes show that the B cells population ($CD3^-$ $CD19^+$) is greatly decreased in TG mice compared to WT mice. **C**, The total number of B cells is decreased in the lymph nodes of TG mice compared to WT mice. **D**, Representative FACS analysis of spleens of WT and TG mice show the relative decrease in follicular B cells ($CD23^{low}$ $CD21^{high}$), while marginal zone B cells ($CD23^{high}$ $CD21^{intermediate}$) proportionally increase. **E**, Absolute quantification of B cells in the spleen shows that both total B cells (B) and follicular B cells (FO) dramatically decrease in the TG mice compared to the WT mice, but the number of marginal zone B cells (MZ) does not change (n = 6 WT vs 6 TG). B = B cells, T = T cells, FO = follicular B cells, MZ = marginal zone B cells, * $p<0.05$, *** $p<0.0005$.

In addition, the relative (data not shown) and absolute numbers (Figure 3E) of total B cells were decreased in the spleen of TG compared to WT mice. We then identified two subpopulations of splenic B cells, namely

follicular (FO) and marginal zone (MZ) B cells characterized by FACS analysis as $CD23^{low}$ $CD21^{high}$ and $CD23^{high}$ $CD21^{intermediate}$, respectively. The decrease in the number to B cells was entirely accounted for by a decrease in the relative and absolute number of FO B cells (Figure 3D and E). In contrast, the number of MZ B cells was relatively increased, while the total number remained constant between WT and TG animals. Of interest, we also noticed that BILF1 expressing TG mice had either no Peyer's patches or a very limited number of small patches in the intestine (data not shown). Interestingly, the development of B cells in bone marrow was comparable in TG mice compared to WT and no significant decrease in B cell numbers was observed (data not shown). Thus, these data demonstrate that BILF1 expression reduces the number of B cells in the periphery, resulting in peripheral B cell lymphopenia.

## B cell organization in the periphery is disturbed in BILF1 transgenic mice

Since BILF1 expression led to a decrease in the number of B cells in the periphery, we examined peripheral lymphoid organs by immunofluorescence. Lymph nodes and spleens sections were stained with anti-B220 and anti-CD3 antibodies to identify B and T cells, respectively. In the organs of WT and TG animals, T cells were surrounded by B cells. The size and repartition of B and T cells were normal in the lymph nodes (Figure 4A) and spleens (Figure 4C) of WT mice. However, the B cell areas in TG mice were dramatically smaller in the lymph nodes (Figure 4B) and spleens (Figure 4D) compared to WT mice, confirming the decrease in B cell numbers observed by flow cytometry. Furthermore, the repartition of splenic MZ and FO B cells was visualized by staining metallotrophic macrophages with anti-MOMA-1 antibodies. This macrophage population separates FO and MZ B cells in the spleen. WT mice presented a normal repartition of FO B cells inside the $MOMA\text{-}1^{+}$ ring, and MZ B cells were located around FO B cells (Figure 4E). In TG spleens, FO B cell follicles were smaller than in WT animals, while the area of MZ B cells was still normal (Figure 4F). Thus, similarly to the FACS analysis, the overall number of B cells was decreased in the spleen and lymph nodes of TG mice, particularly affecting the splenic FO B cell subpopulation.

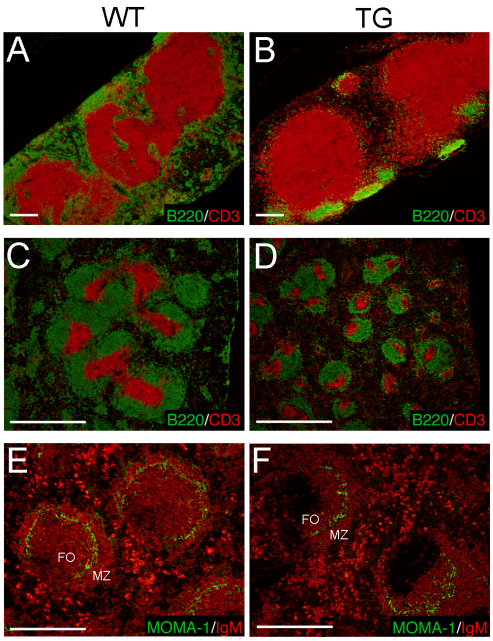


**Figure 4.** BILF1 induces B cell lymphopenia in the lymph nodes and the spleen. **A-B**, Lymph nodes of TG mice (**A**) exhibit a depletion of B cells ($B220^+$ cells) compared to the WT mice (**B**). T cells ($CD3^+$) are present in similar ways in WT and TG animals. **C-F**, Spleen sections of WT (**C**, **E**) and TG (**D**, **F**) mice show that B cells follicles ($B220^+$) are dramatically reduced in TG mice. Follicular (FO) B cells ($IgM^+$ cells inside MOMA-$1^+$ ring) are much decreased in the TG mice, while the marginal zone (MZ) B cells ($IgM^+$ outside MOMA-$1^+$ ring) are not affected. Scale bars represent 250 μm.

## BILF1 expression induces apoptosis of B cells in vivo

As the BILF1 TG mice showed B cell lymphopenia, we investigated whether this could be due to increased apoptosis. To this aim, we determined the percentage of apoptotic cells by using annexin $V^+$ staining. Lymph node B cells from TG mice contained a higher apoptotic fraction than in WT mice, while the number and the extent of apoptosis within T cells in the lymph nodes were unaffected by BILF1 expression (Figure 5A). In the spleen, more B cells were annexin $V^+$ in TG mice compared to WT mice. Both FO and MZ B cells presented an increased apoptotic fraction (Figure 5B). Since Flag-BILF1 is already expressed on the surface of B cells in the bone marrow, we investigated whether increased apoptotic activity could be detected in this organ. We evaluated annexin $V^+$ staining in the different B cell subpopulations that were characterized as B cell precursors ($B220^+$ $CD19^+$ $IgM^-$), immature B cells ($B220^+$ $CD19^+$ $IgM^+$) and recirculating B cells ($B220^{hi}$ $CD19^{hi}$ $IgM^+$). Although WT and TG mice did not present significantly different numbers of B cell subpopulations (data not shown), the apoptotic activity determined by annexin $V^+$ staining in the total B cell subpopulation was approximately doubled in the TG mice compared to WT mice. In particular, TG immature and recirculating B cells showed the most dramatic increases in annexin $V^+$ staining compared to WT mice (Figure

5C). In summary, we measured increased apoptotic activity in TG B cells within all the different B cell maturation organs.

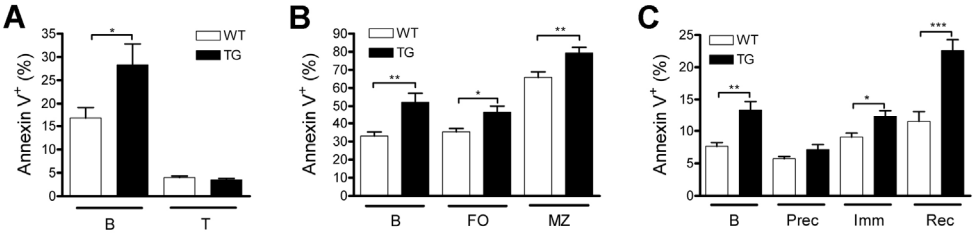


**Figure 5.** BILF1 expression increases apoptosis of B cells in vivo. **A**, Cell surface staining against AnnexinV indicates that TG mice present an increased apoptosis in the B cell compartment of lymph nodes. T cell compartment do not undergo more apoptosis in TG mice compared to WT mice. **B**, In the spleen of TG mice, total B cells (B) as well as the different subclasses of follicular (FO) and marginal zone (MZ) B cells undergo more apoptosis compared to WT mice. **C**, Annexin V staining shows that B cells present more apoptosis in the bone marrow of TG mice than in WT mice, but this is not affecting the $IgM^-$ B cells. B = B cells, Prec = precursor cells, Imm = immature B cells, Rec = recirculating B cells, FO = follicular B cells, MZ = marginal zone B cells, T = T cells. * $p<0.05$, ** $p<0.01$, *** $p<0.0005$.

## BILF1 TG mice present an impaired immune system

Since B cells are responsible for the production of immunoglobulins (Ig), we determined the levels of the different Ig subclasses in the serum of WT and TG mice. Relative amounts of IgM were not significantly different between WT and TG mice. However, all other serum Ig subclasses were significantly diminished in the TG mice compared to the WT mice (Figure 6A). We reasoned that if B cell numbers and Ig titers were decreased in the TG mice, the ability of these mice to mount an immune response against foreign antigens might also be compromised. To address this question, we immunized mice with T cell-dependent (NP-KLH) and T cell-independent (NP-AECM-FICOLL) antigens. Mice were immunized with a single i.p. injection of 50 μg of antigen resuspended in PBS/Alum. Fifteen days post-immunization, mice were sacrificed and the sera analyzed by ELISA. TG mice immunized with the T cell-dependent antigen (NP-KLH) had more than four-fold lower anti-NP antibody titers than WT mice, indicating that the TG mice failed to mount a robust immune response (Figure 6B). However, anti-NP antibody titers after immunization with NP-AECM-FICOLL were comparable between WT and TG mice, showing that T cell-independent responses were unaffected by BILF1 expression. In summary, expression of BILF1 in the B cell compartment impaired the immune response to T cell-dependent antigens, but had no effect on T cell-independent responses.

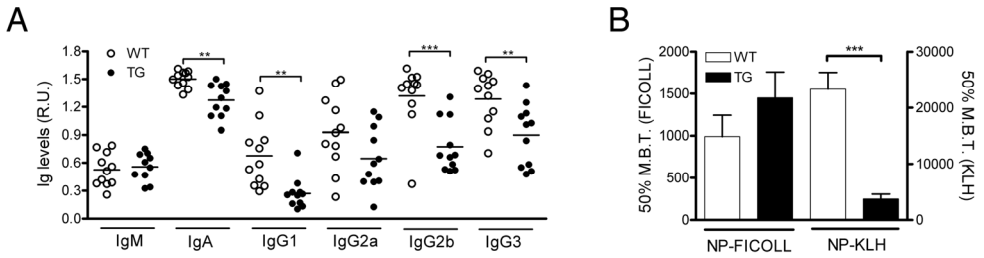


**Figure 6.** BILF1 expressing mice present an impaired immune system. *A*, Titers of the different subclasses of Ig are decreased in TG mice compared to WT, for the exception of IgM. *B*, TG mice immunized with T cell-dependent antigens (NP-KLH) present significantly lower anti-NP antibody titers compared to WT. T cell-independent antigens (NP-FICOLL) do not lead to any impaired anti-NP antibody production. M.B.T., maximal binding titer. ** $p<0.01$, *** $p<0.0005$.

We reasoned that if B cell numbers and Ig titers were decreased in the TG mice, the ability of these mice to mount an immune response against foreign antigens might also be compromised. To address this question, we immunized mice with T cell-dependent (NP-KLH) and T cell-independent (NP-AECM-FICOLL) antigens. Mice were immunized with a single i.p. injection of 50 μg of antigen resuspended in PBS/Alum. Fifteen days post-immunization, mice were sacrificed and the sera analyzed by ELISA. TG mice immunized with the T cell-dependent antigen (NP-KLH) had more than four-fold lower anti-NP antibody titers than WT mice, indicating that the TG mice failed to mount a robust immune response (Figure 6*C*). However, anti-NP antibody titers after immunization with NP-AECM-FICOLL were comparable between WT and TG mice, showing that T cell-independent responses were unaffected by BILF1 expression. In summary, expression of BILF1 in the B cell compartment impaired the immune response to T cell-dependent antigens, but had no effect on T cell-independent responses.

## Discussion

G-protein coupled receptors are fundamental to cellular communication. During their evolution, human herpesviruses have pirated human GPCRs from the infected host (Vischer, Vink et al. 2006). The various vGPCR from HHV present a plethora of functions, ranging from cellular proliferation for the KSHV-encoded ORF74 protein, chemotaxis mediated by the HCMV-derived US28 protein, to chemokine scavenging and immune evasion by US28 and the HHV-6-encoded U51 protein product (Bais, Santomasso et al. 1998; Bodaghi, Jones et al. 1998; Streblow, Soderberg-Naucler et al. 1999; Milne, Mattick et al. 2000). Beta and gamma-HHV are also distinct regarding their implication in proliferation. HCMV is increasingly categorized as an oncomodulator, since it preferably infects and influences cancer cells. HHV-6 and HHV-7 are not recognized as oncogenes. Interestingly, both γ-HHV KSHV and EBV are known oncogenic viruses. Since ORF74, the vGPCR encoded by KSHV, is an important mediator of cellular proliferation in infected cells, it remained interesting to determine if the EBV vGPCR BILF1 possesses similar functions as ORF74. Both KSHV- and EBV-encoded vGPCR are expressed during the lytic phase of infection (Kirshner, Staskus et al. 1999; Beisser, Verzijl et al. 2005). Although the expression of BILF1 in vivo still remains poorly documented, the conservation of the BILF1 gene family in the γ1-herpesvirus subfamily suggested an important role for this viral protein in infected cells (Paulsen, Rosenkilde et al. 2005). Since EBV presents a B cell tropism, we decided to probe the importance of BILF1 in B cell biology by generating TG mice expressing this protein in the B cell compartment. The use of TG animals has widely been used to assess the pathological role of viral proteins (Kulwichit, Edwards et al. 1998; Holst, Rosenkilde et al. 2001; Merchant, Swart et al. 2001; Kang, Soni et al. 2008). To our surprise, our study shows that transgenic mice expressing BILF1 developed a severe peripheral B cell lymphopenia, estimated by the reduced number of B cells in the blood, lymph nodes and spleens. One direct and obvious consequence of the peripheral B cell lymphopenia was the decrease in immunoglobulin titers in the serum of TG mice. Spleens and lymph nodes of TG mice were depleted of B cells and consequently, titers of IgGs released in the circulation were reduced. Interestingly, the levels of IgA and IgG in serum were decreased but the IgM levels were normal (Figure 5A and 2F). The unaltered numbers of IgM-producing B cells, i.e. immature B cells, MZ B cells and recirculating B cells, in TG mice were most likely responsible for the normal production of serum IgM antibodies (Cariappa, Mazo et al. 2005). Finally, we showed that immune responses are compromised in TG mice (Figure 5C). T cell-dependent immune responses are mounted by the FO B cells and T cell-independent responses by the MZ

B cells (McHeyzer-Williams 2003). TG mice expressing BILF1 have similar numbers of MZ B cells whereas FO B cells are dramatically decreased, providing a rational for the impaired immune response against the T cell-dependent antigen only.

Our findings suggest that the cause of B cell lymphopenia in TG animals is increased apoptotic activity. Lymphocytes from the bone marrow, spleen and the lymph nodes had a higher expression of annexin V, which may be the consequence of signaling pathways activated by BILF1. *In vitro* studies using transfection of BILF1 have shown that NF-κB and CRE-related signaling are differentially altered in two different cell types. EBV-negative COS-7 cells showed BILF1-induced activation of transcriptional activity. Besides promoting survival of lymphocytes during lymphopoiesis, NF-κB also plays a role in the programmed cell death of lymphocytes (Goudeau, Huetz et al. 2003). Mice deficient in the inhibitory subunits of the NF-κB transcriptional complex, IκBα and IκBε, have increased NF-κB activity and impaired B cell development (Goudeau, Huetz et al. 2003). These mice have very low numbers of $IgM^+$ B cells and increased apoptosis of B cells. Therefore, we could envision that the constitutive activity of BILF1 may alter NF-κB signaling pathways that would favor B cell apoptosis rather than B cell proliferation.

The apparent divergent functions of γ-HHV-encoded vGPCRs are intriguing. Both the ORF74 and BILF1 genes are lytic genes and signal in a constitutive manner. However, while the KSHV-encoded ORF74 gene is implicated in cellular proliferation *in vitro* and *in vivo* (Arvanitakis, Geras-Raaka et al. 1997; Yang, Chen et al. 2000), the EBV-derived BILF1 gene induces apoptosis in vivo. An in vivo infection model system used to mimic the effects of γ-HHV infection was created with the γ-herpesvirus murine herpesvirus-68 (MHV-68). Immunocompromised mice infected with MHV-68 develop a proliferative disease that resemble the EBV-associated post-transplant proliferative disorders observed in human transplant recipients (Tarakanova, Suarez et al. 2005). Similarly to KSHV and EBV, MHV-68 encodes a single vGPCR. Recently, it was shown that this vGPCR is dispensable for the formation of lymphoproliferative diseases (Tarakanova, Kreisel et al. 2008). Mice infected with a mutant MHV-68 virus lacking this single vGPCR gene developed diseases to a similar extent than mice infected with the WT virus. As such, the MHV-68-encoded vGPCR does not present proliferative properties, indicating that not all vGPCRs from γ-herpesvirus induce proliferation, and that this property is dependent on each single receptor. The low homology between ORF74 and BILF1 and their different signaling abilities may be serve as rational for their different functions. Sequence alignment studies showed that BILF1 and

ORF74 only share 15% amino acid homology, establishing them into two different classes of vGPCRs (Beisser, Verzijl et al. 2005; Zuo, Currin et al. 2009). In addition, KSHV-encoded ORF74 has been shown to activate multiple G proteins, e.g. $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{13}$ and $G\beta\gamma$ (Shepard, Yang et al. 2001; Smit, Verzijl et al. 2002), while until now only $G\alpha_{i/o}$ proteins were demonstrated to be constitutively activated by BILF1 (Beisser, Verzijl et al. 2005). Although it may simply be that vGPCRs from different classes possess different functions, proteins from the same vGPCR family do not necessarily present similar implications in proliferative diseases. This is the case for both MHV-68- and KSHV-encoded vGPCR, which belong to the same ORF74 gene family (Beisser, Verzijl et al. 2005). Both MHV68- and KSHV-encoded ORF74 genes bind chemokines, but only ORF74 possesses constitutive activity (Verzijl, Fitzsimons et al. 2004). Importantly, while KSHV-ORF74 has been implicated in proliferative processes in vivo, the MHV-68-derived vGPCR gene seems to be dispensable for the development of virally-induced lymphoproliferative diseases (Yang, Chen et al. 2000; Tarakanova, Kreisel et al. 2008).

Our study indicates a role for BILF1 during the lytic phase of infection. In EBV-infected B cells, the switch from latent to lytic infection induced by the viral immediate early protein BZLF1 results in death of the host cells (Westphal, Mauser et al. 1999). Interestingly, chromosomal DNA fragmentation of lytically infected B cells has been attributed to an EBV-specific early protein (Kawanishi 1993). Since BILF1 is an immediate early or early viral gene, our findings may provide a role for this vGPCR in the apoptotic processes in lytically infected B cells. In addition, it was recently shown that BILF1 expression downregulates MHC class I expression, facilitating immune escape of cells producing EBV particles. This implies that BILF1 may have several functions during lytic infection to facilitate virus production. The BILF1-induced subversion of the immune system allows cells producing EBV proteins and particles to remain undetected by the immune system. The BILF1-induced apoptosis may occur at a later stage once lytic replication is fulfilled, resulting in the shedding of EBV particles. Furthermore, BILF1 was recently shown to heterodimerize with a variety of human chemokine receptors, and this may also have an influence on the localization/migration abilities of the infected cells (Vischer, Nijmeijer et al. 2008).

Taken together, our results show that expression of the constitutively active EBV-encoded GPCR BILF1 in B cells of TG mice surprisingly results in severe peripheral lymphopenia. This phenotype was not due to an abnormal B cell development but to an increased apoptotic activity of the B cells. This resulted in an impaired immune system of the BILF1-

expressing mice. As such, in homeostatic conditions, BILF1 seems to present pro-apoptotic properties *in vivo*.

## Materials and Methods

**Cell culture.** African green monkey COS-7 cells, human glioblastoma U373 cells and mouse fibroblast NIH-3T3 cells were cultured in DMEM supplemented with 10% of fetal calf, heat inactivated fetal calf and calf sera, respectively. Transfections were performed in

**Generation of mice.** A N-terminal FLAG-epitope tag (DYKDDDDK) was introduced after the initial startcodon of BILF1 (EBV strain B95-8). FLAG-BILF1 displayed similar signaling properties as WT wild type BILF1 when transiently transfected in HEK293T cells (Vischer et al. unpublished data). Next, appropriate restriction sites were inserted by PCR using the following forward 5'-CAG GCG CGC CAC CAT GGA CTA CAA GGA C-3' and reverse 5'-CGT TAA TTA ATC AGG TGG ACT GGC TAG G-3' primers and FLAG-BILF1/pcDNA3.1 as template. The PCR product was sequence verified and subsequently subcloned with AscI and PacI into an Igκ expression vector initially containing GFP (Robbiani DF, unpublished). Igκ is a derivative of the Vk vector and includes: the 5' portion of the Vk21E gene, unique AscI and PacI restriction sites, the 3'UTR of the mouse kappa light chain gene, the kappa intronic sequence with enhancer, and the kappa 3' regulatory region (Robbiani, Colon et al. 2005). The transgene was cleaved by MluI and NotI and microinjected into [C57BL/6J x DBA/2]F1 (The Jackson Laboratory, Bar Harbor, Maine) eggs and transferred into oviducts of ICR foster mothers (Charles River Laboratories, Wilmington, Mass.) according to published procedures. Identification of transgenic animals was done by PCR amplification of tail DNA. Primers used for the detection of the BILF1 gene and the ZIP3 housekeeping gene were BILF1 forward 5'-GGC GTG TTT TGT CTG GTA CT-3', BILF1 reverse 5'-GGA CAG CAT AAA CCA GGA GA-3', ZIP3 forward 5'-CAG CTC TAC ATC ACC TGC CA-3', ZIP3 reverse 5'-CAC TGG GAA GAG ACA CTC AG-3'. Transgenic mice were kept under pathogen-free conditions. All experiments involving animals were performed following the guidelines of the Mount Sinai School of Medicine Animal Care and Use Committee. Mice used in the different experiments were between 8 and 10 weeks of age.

**Gene expression analysis by quantitative PCR.** Total RNA from the studied organs was extracted using the RNeasy Maxi Kit (Qiagen), according to the manufacturer's instructions. RNA quality was measured using Agilent Bioanalyzer (Agilent Technologies). For cDNA synthesis, we followed previously described procedures (Jensen, Manfra et al. 2005). Primers used to detect BILF1 and the housekeeping gene Ubiquitin were BILF1 forward 5'-GCT CTG GGT GCT GGG AAA-3', BILF1 reverse 5'-CAA AAG GCA GAG ACC GGT AGA-3', Ubiquitin forward 5'- TGG CTA TTA ATT ATT CGG TCT GCA T-3', Ubiquitin reverse 5'- GCA AGT GGC TAG AGT GCA GAG TAA-3'.

**Lymphocyte preparation and FACS analysis.** Bone marrow cells were obtained by flushing femurs and tibia of mice with DMEM containing 2% fetal bovine serum and passed through a 100-μm nylon cell strainer. Cells from spleen and lymph nodes were obtained by gently squeezing the organs on a 100-μm nylon cell strainer. After washing and lysis of the red blood cells with ACK lysis buffer, cells were stained with different cocktails of antibodies. Bone marrow cells were stained anti-IgM-APC (BD Biosciences, 550676), anti-CD19-APC-Cy7 (BD Biosciences, 55765), anti-CD25-PE (eBioscience, 12-0251), anti-B220-PE-Cy7 (eBioscience, 25-0452). Spleen cells were stained using anti-IgM-APC, anti-CD19-APC-Cy7, anti-CD21-PE (eBioscence, 12-0211), anti-CD23-PE-Cy7 (eBioscience, 25-0232). Cells from the lymph nodes were stained using anti-CD19-APC-Cy7, anti-CD3-APC (eBioscience, 17-0031-82). Blood analysis was performed using anti-B220-APC (BD Biosciences, 01129A), anti-CD3-PE (eBioscience, 12-0031-82) and anti-Flag-FITC (Sigma, F4049). For the apoptosis experiments, anti-annexin V-PE (BD, 556422) replaced CD25-PE

in the bone marrow cocktail, and anti-annexin V-PE was supplemented into the lymph node cocktail. For the spleen analysis, anti-annexin V-biotin (BD Biosciences, 556418) was used in the spleen cocktail above and secondly labeled with FITC-labeled streptavidin (BD Biosciences, 554060).

**Immunohistochemistry.** Frozen sections of spleen or lymph nodes were fixed with ice-cold acetone for 20 min, dried and then stored at –20°C. For the staining, slides were rehydrated in PBS, blocked with 10% BSA and incubated for 1 h at room temperature with purified primary antibodies. Specific staining was determined by using the appropriate labelled secondary antibody for 30 min. The primary Abs used were anti-B220 (BD Biosciences, 550286), anti-CD3 (BD Biosciences, 553058), anti-IgM (eBiosciences, 14-5790-82), anti-MOMA-1 FITC conjugated (Serotec, MCA947F). The secondary antibodies used were Alexa Fluor 488 goat anti-rat IgG (Molecular Probes, A-11006), Alexa Fluor 594 goat anti-rat IgG (Molecular Probes, A-11007), and Cy3 goat anti-armenian hamster (Jackson Immunoresearch, 127-165-160). Fluorescence images were acquired using a Nikon Eclipse E600 microscope system with Nikon 4x, 10x 20x and 40x Plan Fluor Objective, captured with a Nikon DXM 1200F camera and processed with the Nikon ACT-1 software.

**Ig ELISA.** Immunoglobulins subclasses from the serum of mice were qualitatively measured using the mouse immunoglobulin isotyping ELISA kit from BD Pharmingen (550487). ELISA was performed according to the manufacturer's procedure. IgA ELISA was measured from resuspended fecal pellets as previously described (Shang, Fukata et al. 2008).

**Immunization.** Mice were immunized intraperitoneally with 200 μl of a mixture PBS:Alum (Pierce) 1:1 containing 50 μg nitrophenyl NP(28)-keyhole limpet hemocyanin (NP(28)-KLH; Biosearch Technologies, N-5060-5) or nitrophenyl NP(41)-AECM-FICOLL (Biosearch Technologies, F-1420-10). Serum was obtained two weeks following immunization. Levels of NP-binding antibodies were measured in the sera of mice by ELISA on plates coated with 5 μg/ml NP(23)-BSA (Biosearch Technologies, N-5050-10) in PBS. Bound Ab was detected using 0.2 μg/ml AP-conjugated anti-mouse IgG Ab (Caltag Laboratories), followed by p-nitrophenyl phosphate (Sigma-Aldrich) at 1 mg/ml. OD of the wells was measured at 405nm. In order to compare the different samples, serial dilution curves were used to calculate the 50% maximal binding titer (dilution factor giving 50% of the maximal OD measured for each sample).

# VII

Discussion and future perspectives

# I. Advantages of human GPCR hijacking by herpesviruses

## Evolution of herpesviruses and hijacking of chemokine receptors

During evolution, mammalian herpesviruses have coexisted with their natural host and subsisted until now. The different subfamilies of herpesviruses, namely the alpha-, beta- and gamma-herpesviruses are thought to have arisen approximately 180 to 220 million years ago and to have evolved along with their hosts (McGeoch, Cook et al. 1995). In order to coexist within the infected organism and avoid being eliminated, viruses have acquired some of their host's genes and integrated them into their genome. It is believed that this occurred during host's coinfection with herpesviruses and retroviruses. In order to replicate, retroviruses integrate their genetic information into the host genome so that the host transcriptional machinery transcribes viral information. When inserted near a host gene, chimeric transcripts of human and viral information are occasionally generated and can be packaged into the forming retroviral virions (Brunovskis and Kung 1995). In addition, it was shown that during coinfection of retroviruses and herpesviruses, genetic information from the retrovirus integrates into the herpesvirus genome (Isfort, Jones et al. 1992). As such, retroviruses are thought to shuffle genetic material between the host and the herpesvirus and to be responsible for the hijacking of G protein-coupled receptors descending from host's chemokine receptors (Brunovskis and Kung 1995).

## Implication in immune evasion and viral diseases

Upon entry into infected cells, herpesviruses exploit the host transcriptional machinery in order to efficiently replicate and spread within the infected host. However, viruses also integrated genes derived from their human hosts in order to persist. During evolution, the most beneficial genes for viruses have been conserved, explaining why nowadays we find in viral genomes several cellular homologues to genes involved in the regulation of the immune system (Vischer, Vink et al. 2006). Particularly, the chemokine system is crucial for the correct functioning of the immune system (Mackay 2001) and viral chemokine receptors play a prominent role during immune evasion. Although human chemokine receptors signal in a ligand-dependent manner, viruses have reshaped the acquired human genes to render them constitutively active in some cases.

As previously exemplified for HCMV-US28 and HHV-6A-U51, binding and subsequent removal of chemokines from the extracellular medium impair the local inflammatory microenvironnement surrounding the infected cell and reduce immune surveillance (Bodaghi, Jones et al. 1998; Milne, Mattick et al. 2000). U51 expression also downmodulates the expression of the inflammatory chemokine CCL5 (Milne, Mattick et al. 2000). In addition, constitutive activity of vGPCRs can directly affect the transcriptional regulation of genes involved in recognition and presentation of viral antigens during immune responses. For instance, the constitutively active EBV-encoded vGPCR BILF1 has recently been shown to downmodulate MHC Class I molecules, reducing recognition of infected cells by CD8+ T cells (Zuo, Currin et al. 2009). This may represent a way for the virus to escape from the immune system.

In addition, the involvement of vGPCRs in viral pathologies has been best exemplified with the KSHV-encoded chemokine receptor ORF74. Almost simultaneously to its discovery, ORF74 was found to bind human chemokines and induce proliferation (Cesarman, Nador et al. 1996; Arvanitakis, Geras-Raaka et al. 1997). In the next following two years, it became clear that in an in vivo setting, ORF74 was also leading to the formation of tumors resembling lesions observed in KS patients (Bais, Santomasso et al. 1998; Yang, Chen et al. 2000). As such, ORF74 was shown to play a prominent role of the development of KSHV-related diseases. Although many vGPCR present constitutive activity, their involvement in pathologies is not similar and within this thesis, we further identified new roles for the EBV-encoded GPCR BILF1 and the HCMV-encoded chemokine receptor US28.

## II. BILF1, a key player during lytic replication

### *In vitro* evidence of immune evasion

EBV encodes a single viral GPCR, BILF1, which is expressed during the lytic phase of infection (Beisser, Verzijl et al. 2005; Kaptein, Jungscheleger-Russell et al. 2008). Although initially considered as a chemokine receptor, extensive binding and signaling studies failed to demonstrate the binding of human chemokines to BILF1. In addition, there is only limited information on the physiological function of BILF1. We initially showed constitutive signaling activity for this vGPCR. When expressed in COS-7 and immortalized B cells, BILF1 modulated the transcriptional activation of the NF-κB and CREB transcription factors (Beisser, Verzijl et al. 2005). The modulatory effects were different in

epithelial and B cells. In particular, NF-κB activation was only observed in COS-7 cells and not B cells. This apparent non-activation of NF-κB in immortalized B cell lines is thought to be due to the presence of latent EBV. During latency, the LMP1 gene is known to strongly activate NF-κB signaling (Eliopoulos and Young 2001), therefore hindering detection of BILF1 constitutive activation towards NF-κB.

Two *in vitro* studies indicate that BILF1 possesses immune evasion functions. Our laboratory showed that expression of BILF1 constitutively inhibits the phosphorylation of RNA-dependent protein kinase (PKR) (Beisser, Verzijl et al. 2005). Upon viral infection, double strand RNA bind to PKR, leading to its phosphorylation and activation. As a result, the overall cellular transcriptional machinery is stopped, prohibiting viral replication, and only a few specific apoptotic genes are transcribed (Garcia, Gil et al. 2006). This mechanism serves to prevent viral spreading by elimination of the infected cells. Thus, the inhibition of PKR by BILF1 may serve EBV to prevent cellular anti-viral response. In addition, Zuo et al. recently demonstrated that BILF1 expression also serves in immune escape by downregulating antigen-presenting MHC Class I cells of epithelial and melanoma cells (Zuo, Currin et al. 2009). BILF1 protein physically associated with MHC class I complexes, increasing their lysosomal degradation and downregulating their surface expression. This mechanism is independent from constitutive G protein coupling since the BILF1 mutant K122A, unable to constitutively activate NF-κB, downmodulated MHC I surface expression to a similar extent than the WT receptor (Zuo, Currin et al. 2009). As such, BILF1 expression was shown to inhibit CD8+ T cell recognition, preventing recognition by the host immune system.

### *In vivo* evidence of apoptosis

Gamma-herpesviruses are known to possess transforming properties and are involved in various proliferative diseases (Damania 2004). As previously described, the γ2-HHV KSHV encodes for a single VGPCR, the lytic gene ORF74, which induces cellular proliferation and transformation. ORF74 is regarded as a molecular mediator of viral transformation. We investigated whether BILF1 played a similar role in EBV-related diseases. Similarly to ORF74, it is the only vGPCR encoded by the γ1-HHV EBV and it is also a lytic gene. To this aim, we generated an in vivo transgenic (TG) expression model where BILF1 expression was restricted to the B cell compartment (**Chapter 6**). This cellular target was chosen based on the B lymphocyte tropism of EBV (Maussang, Furtado et al. In preparation). The generated TG mice developed normally, but to our surprise, the BILF1-expressing mice

exhibited a profound B cell lymphopenia in all their peripheral compartments. The number of B cells was greatly diminished in lymph nodes, spleen, blood and Peyer's patches in the small intestine. Noteworthy, the composition of the bone marrow appeared unchanged between TG and wild type (WT) animals. During the characterization of BILF1-induced peripheral lymphopenia, we showed that splenic follicular B cells were less numerous in TG mice compared to WT mice, while marginal zone B cells were not affected. In line with this observation, BILF1 TG mice were unable to mount a proper immune response against the T cell-dependent antigen keyhole limpet hemocyanin (KLH), but they could respond to the T cell-independent antigen FICOLL. Since follicular and marginal zone B cells are respectively responsible for T cell-dependent and T cell-independent immune responses, we attributed the immunocompromised phenotype to the abnormal repartition of splenic B cells subtypes. Annexin V staining showed that both the central and peripheral B cell compartments of TG animals presented a higher apoptotic activity compared to WT mice (Maussang, Furtado et al. In preparation). This finding remained surprising due to the unchanged cellularity of the bone marrow and may reflect some altered migration abilities in the B cells from the TG animals. Lymphocyte migration is mediated in part by chemokine receptor expression. Their expressions vary with the different maturation or activation stages, enabling cells to respond in distinctive manners and migrate towards specific chemokine-secreting sites (Cyster 2005). In addition, the egress of lymphocytes from various lymphoid organs relies on the expression/repression of sphingosine 1 phosphate (S1P) receptors and the interaction with S1P present in the circulation. Interestingly, our laboratory has recently demonstrated that BILF1 heterodimerizes with a great variety of chemokine receptors (Vischer, Nijmeijer et al. 2008). The effects of heterodimerization are numerous and can amongst others affect receptor signaling and response towards chemokines (Salanga, O'Hayre et al. 2008). As such, one can envision that heterodimerization of BILF1 with other chemokine receptors may negatively affect the migration ability of TG B cells and lead to their retention in the bone marrow. One can also not exclude the physical interaction of BILF1 with S1P receptors, which may also alter the egress properties of the cells. Taken together, data from our in vivo model indicate that, unlike the KSHV-encoded vGPCR ORF74, BILF1 is not involved in cellular proliferation and viral proliferative diseases since its expression results in cellular death in vivo in immunocompetent EBV-free animals.

### Dual functions of BILF1 during lytic viral replication

BILF1 expression is restricted to the lytic phase of infection. Interestingly, activation of the BZLF1 gene in latently EBV-infected cells induces lytic viral replication, resulting in cellular death (Westphal, Mauser et al. 1999). As such, our in vivo data supports a role for the lytic BILF1 gene in cellular apoptosis during lytic replication (Maussang, Furtado et al. In preparation). In addition, the recent study from Zuo et al. indicates that BILF1 serves in evading the immune system during viral lytic replication (Zuo, Currin et al. 2009). The authors demonstrate that this immune evasion function may be common to other BILF1 genes that are highly conserved amongst $\gamma$1-herpesviruses (Paulsen, Rosenkilde et al. 2005). Both *in vitro* and *in vivo* findings suggest that BILF1 function may depend on its temporal and/or quantitative expression. At the beginning of lytic replication, the virus needs to avoid immune surveillance to fully replicate and produce infectious particles. By expressing BILF1, EBV downregulates MHC class I expression and evades CD8+ T cell recognition and immune surveillance. At a later stage during lytic replication, the virus may benefit from the host cell death by being released more easily from the cytoplasm and spread through the host. At this point, BILF1 and most likely other lytic genes would participate in the induction of cellular death.

## III. US28 constitutive activity and oncogenesis

### US28, discoveries leading to the identification of an oncogenic potential

US28 is the best-characterized HCMV-encoded vGPCR. First identified as a potential GPCR in the AD169 strain of HCMV (Chee, Satchwell et al. 1990), US28 was subsequently characterized as a chemokine receptor and a potential way for the virus to escape immune surveillance. HCMV infection was shown to reduce the levels of chemokines present in the supernatant of infected cells. Genetic deletion of the US28 gene from the HCMV genome impaired chemokine scavenging (Gao and Murphy 1994; Kuhn, Beall et al. 1995; Billstrom, Johnson et al. 1998). In addition, due to its homology to chemokine receptors, US28 was discovered as a co-receptor for HIV. In CD4+ T cells, US28 was able to mediate the entry of HIV strains known to use the human chemokine receptors CCR5 or CXCR4 as coreceptor (Pleskoff, Treboute et al. 1997). Two years later, the involvement of US28 in HCMV-related

cardiovascular pathologies was established (Streblow, Soderberg-Naucler et al. 1999). Infection of smooth muscle cells with HCMV increased cellular migration. Interestingly, the US28 gene product was shown to induce chemotaxis in transfected and HCMV-infected cells. As such, the chemokine-mediated stimulation of US28 was proposed to be a molecular link between HCMV infection and the development of vascular diseases, e.g. atherosclerosis (Streblow, Soderberg-Naucler et al. 1999).

A decade after its identification as a vGPCR, our laboratory showed that US28 signals in a ligand-independent, i.e. constitutive, manner (Casarosa, Bakker et al. 2001). Around this period, constitutive activity of the KSHV-encoded ORF74 was shown to be responsible for the proliferation and formation of KS-like lesions in TG animals (Arvanitakis, Geras-Raaka et al. 1997; Bais, Santomasso et al. 1998; Yang, Chen et al. 2000). Studies highlighting signaling similarities between both vGPCRs led us to investigate the role of US28 also in a proliferative/oncogenic context (McLean, Holst et al. 2004). In **chapter 3** of this thesis, we identified a tumorigenic potential for the HCMV-encoded chemokine receptor US28. We stably expressed US28 in NIH-3T3 cells and the resulting cell line presented an increased proliferation, loss of cell contact inhibition and production of the angiogenic factor VEGF. More detailed signaling studies in transiently transfected cells demonstrated that the constitutive coupling to $G\alpha_q$ and $G\beta\gamma$ subunits was responsible for the activation of the VEGF promoter gene. When we injected US28-expressing NIH-3T3 cells into nude mice, tumors developed within 3 weeks post injection, demonstrating the oncogenic potential of US28 (Maussang, Verzijl et al. 2006).

## Importance of the cellular background

The inability of US28 to transform all cell lines raises questions on the importance of cellular context. The fact that NIH-3T3 cells were transformed upon expression of US28 does not strictly classify US28 as an oncogene. In fact, previous studies have shown that transfection of US28 into the human epithelial HEK 293T and HeLa cell lines resulted in apoptosis (Pleskoff, Casarosa et al. 2005). To rule out artifacts due to the transfection methods, human chemokine receptors were compared to US28 and failed to induce apoptosis. As such, the cellular context seems to influence US28 behavior. The importance of cellular context for viruses and viral proteins is not novel. For instance, HCMV is known to infect a broad range of cells e.g. endothelial, epithelial and fibroblast cells, but this process is highly dependent on the expression of cellular receptors, such as integrins, epidermal growth factor and platelet-

derived growth factor receptors (Wang, Huong et al. 2003; Wang, Huang et al. 2005; Soroceanu, Akhavan et al. 2008). Similarly, the cellular responses induced by the KSHV-encoded chemokine receptor ORF74 have been shown to be cell type-specific. In particular, the secretion of soluble factors appeared to be depending on the target cell. The common characteristic of endothelial, epithelial, lymphocytic and monocytic cells expressing ORF74 is the constitutive activation of the NF-κB transcription factor (Pati, Cavrois et al. 2001; Schwarz and Murphy 2001; Polson, Wang et al. 2002; Pati, Foulke et al. 2003; Martin, Galisteo et al. 2008). However, the resulting profile of secreted pro-inflammatory factors differed between cells. ORF74-expressing endothelial cells released CCL5, CXCL1, CXCL8, IL-6 and VEGF-C (Pati, Cavrois et al. 2001; Polson, Wang et al. 2002), while epithelial cells released bFGF, CCL2 and CXCL8 (Schwarz and Murphy 2001). Also, T and B cells presented distinct profiles of ORF74-induced secreted proteins with increased levels of IL-2, IL-4, GM-CSF, TNFα (Schwarz and Murphy 2001; Pati, Foulke et al. 2003) and CCL3, CCL4 (Polson, Wang et al. 2002), respectively. As such, this indicates that different cellular backgrounds respond differently to ORF74 expression.

The activation of a single signaling pathway, e.g. a common transcription factor, in different cells by the same vGPCR may also result in opposite phenotypes. For instance, the so-called "NF-κB" is a complex system which integrates proteins from the NF-κB and IκB families (composed of 5 and 7 members, respectively), and are regulated by IκB kinases (IKK, corresponding to 3 different enzymes) (Hayden and Ghosh 2004). In an inactive state, NF-κB dimers are bound to IκB proteins and remain in the cytoplasm. Extracellular signals can activate IKK that will phosphorylate the IκB subunit, leading to either its degradation or its processing giving rise to NF-κB subunits. As a result, the released or newly-generated NF-κB dimers translocate into the nucleus and initiate gene transcription (Hayden and Ghosh 2004). Furthermore, nuclear kinases, co-activators and repressors of the translocated NF-κB dimer can greatly promote or repress its transcription ability and provide different cellular responses (Perkins and Gilmore 2006). NF-κB is typically classified as a pro-inflammatory transcription factor involved in cellular proliferation. In particular, it has been involved in tumor formation and metastasis due to its ability to induce anti-apoptotic genes, adhesion molecules, angiogenic factors, proto-oncogenes and inflammatory cytokines. However, in other conditions, NF-κB is also responsible for cellular damage and subsequent cell death. For instance, this transcription factor can activate pro-apoptotic genes such as Fas, induce cell cycle arrest-related genes such as p21 or contribute to p53-induced apoptosis. (Perkins and Gilmore 2006). Thus, this indicates that

the activation of NF-κB transcription factor may lead to different phenotypes in different cell types.

**Importance of constitutive receptor activation (Table 1)**

The fact that US28 constitutive activity was sufficient to induce tumor formation in nude mice correlates with the development of diseases due to constitutively active mutants of human GPCR (Seifert and Wenzel-Seifert 2002). The importance of constitutive activity in proliferation was initially discovered after the introduction of point mutations in the $\alpha_{1B}$ adrenergic receptor (Allen, Lefkowitz et al. 1991). Mutations in the third intracellular loop led to a constitutively active receptor that induced cellular transformation *in vitro* and in a xenograft model. This has since then stimulated a broad field of research on naturally occurring mutations of human GPCR and the generation of inverse agonists against constitutively active mutants may represent novel therapeutic approaches (Seifert and Wenzel-Seifert 2002). In addition, the interaction partners of GPCR, i.e. the G proteins themselves, are also prone to genetic mutations leading to pathological conditions. Mutationally activated proteins from the $G\alpha$ family, e.g. $G\alpha_s$ and $G\alpha_i$, have been identified in different malignancies, e.g. pituitary glands, thyroid and adrenal cortex tumors (Radhika and Dhanasekaran 2001). The $G\alpha_q$ subunit is known to activate proliferative signaling pathways (Radhika and Dhanasekaran 2001), and it was recently discovered that mutation of codon 209 in the GNAQ gene was frequent in melanocytic neoplasias (Van Raamsdonk, Bezrookove et al. 2009). The resulting mutant $G\alpha_q$ protein is constitutively active and acts as on oncogene.

The concept of "constitutive activity" goes beyond the scope of G proteins and their receptors. For instance, genetic point mutations leading to the constitutive activation of kinases (e.g. Janus activated kinase 2 JAK2 (Levine and Gilliland 2007)) and receptor tyrosine kinases (RTKs) (e.g. the cytokine receptor for prolactin (Bogorad, Courtillot et al. 2008)) have been described in proliferative diseases. Also, genomic rearrangements and splicing variants can lead to the generation of constitutively active chimeric oncogenes and deletion mutants of RTKs, respectively. This was recently shown for the nerve growth factor receptor. Papillary thyroid tumor and neuroblastoma were found to express constitutively active mutants of this RTK, leading to cellular proliferation (Pierotti and Greco 2006). Chromosomal translocation can result in fusion proteins that possess constitutive activity. One such example is the creation of the BCR-ABL protein that exhibits constitutive tyrosine kinase activity and is involved in the development of chronic myeloid leukemia (Saglio and Cilloni 2004). Mutations of negative

regulators of signaling networks can observe a loss of function, resulting in the constitutive activation of the corresponding signaling pathway. For instance, the phosphatase and tensin homolog (PTEN) and the inhibitory IκB subunit are negative regulators of PI3K and NF-κB signaling pathways, respectively. Silencing mutations of PTEN and IκB have been found in proliferative diseases, providing a molecular mechanism for constitutive activation of PI3K and NF-κB signaling pathways in brain tumors and lymphoproliferative disorders (Rayet and Gelinas 1999; Endersby and Baker 2008). As such, "constitutive activity" appears to be an important component in proliferation and transformation.

## Is the G protein-uncoupled mutant US28-R$^{129}$A really inactive?

An important motif for the activation of G proteins by US28 is the DRY sequence present in the second intracellular loop (Rosenkilde, Smit et al. 2008). Previous results from Casarosa et al. and characterization of US28 in inflammatory and proliferative pathways failed to show any signaling activity for the US28-R$^{129}$A mutant. Reporter genes for the activation of the NF-κB and STAT3 transcription factors, as well as for the transcriptional regulation of the VEGF and COX-2 genes indicated that this point mutation resulted in a silent vGPCR (Casarosa, Bakker et al. 2001; Maussang, Verzijl et al. 2006; Maussang, Langemeijer et al. 2009; Slinger, Maussang et al. In preparation). However, to our surprise, NIH-3T3 cells stably expressing US28-R$^{129}$A were still able to induce tumor formation in our xenograft model, although at a later time that the WT receptor. This finding appeals for reconsideration of the apparent silence of US28-R$^{129}$A and opens up new angles to examine G protein-independent signaling properties of US28.

Our *in vitro* characterization of US28-R$^{129}$A signaling activities has usually been performed in short-term assays, usually 24h post-transfection in the absence of ligands. Such assays have always failed to show activation of transcription factors such as NF-κB (Casarosa, Bakker et al. 2001), STAT3 (Slinger, Maussang et al. In preparation) and NFAT (Slinger, unpublished observations), as well as the inflammatory protein COX-2 (Maussang, Langemeijer et al. 2009) and the angiogenic factor VEGF (Maussang, Verzijl et al. 2006). However, looking back at some published data, we may find subtle evidence of US28-R$^{129}$A signaling abilities. On figure 2 of **chapter 3**, the proliferation index of US28-R$^{129}$A is actually slightly higher than mock-transfected cells, which was also accompanied by a minor increase in Cyclin D1 expression levels.

**Table 1.** Constitutively activated proteins or signaling pathways in human diseases

| Protein | Clinical phenotype, disease | Reference |
|---|---|---|
| *GPCR* | | |
| Rhodopsine | Congenital blindness, retinitis pigmentosa | Robinson, Cohen et al. 1992; Dryja, Berson et al. 1993 |
| Cholecystokinin B receptor (CCKBR) | Gastric carcinoid tumors | Kopin, McBride et al. 2000 |
| Thyroid stimulating hormone receptor (TSHR) | Adenoma or hyperplasia associated with hyperthyroidism | Seifert and Wenzel-Seifert 2002 |
| Luteinizing hormone receptor (LHR) | Male precocious puberty | Seifert and Wenzel-Seifert 2002 |
| Follicle stimulating hormone receptor (FSHR) | Normal semen parameters despite hypohysectomy | Gromoll, Simoni et al. 1996 |
| Parathyroid hormone 1 receptor (PTH1R) | Jansen-type metaphyseal chondrodysplasia (dwardfism, hypercalcemia, hypophosphatemia) | Schipani, Kruse et al. 1995 |
| Calcium sensing receptor (CASR) | Autosomal dominant hypocalcemia | Zhao, Hauache et al. 1999 |
| *G protein* | | |
| $G\alpha_s$ | Pituitary tumors, thyroid adenomas and thyroid carcinomas | Radhika and Dhanasekaran 2001 |
| $G\alpha_i$ | Pituitary tumors, ovarian sex cord stromal tumors and adrenal cortex tumors | Radhika and Dhanasekaran 2001 |
| $G\alpha_q$ | Melanocytic neoplasias | Van Raamsdonk, Bezrookove et al. 2009 |
| *Kinase-related* | | |
| JAK2 | Myeloproliferative disorders, e.g. polycythemia vera, thrombocytosis | Levine and Gilliland 2007 |
| RTK prolactin receptor | Multiple fibroadenoma (benign breast tumor) | Bogorad, Courtillot et al. 2008 |
| RTK nerve growth factor receptor | Pappilary thyroid tumors, colon cancer | Pierotti and Greco 2006 |
| BCR-ABL | Chronic myeloid leukemia | Saglio and Cilloni 2004 |
| *Negative signaling regulator (signaling pathway)* | | |
| PTEN (PI3K) | Brain tumors | Endersby and Baker 2008 |
| IκB (NF-κB) | Lyphoproliferative disorders | Rayet and Gelinas 1999 |

Furthermore, the study of Bakker et al. on the effect of cotransfection of $G\alpha_q$- and $G\alpha_i$-coupled GPCRs may imply minor signaling induced by the US28-$R^{129}A$ mutant in a ligand-stimulated setting (Bakker, Casarosa et al. 2004). CCL5 incubation of cells cotransfected with CCR1 and US28-$R^{129}A$ presented a slight activation of NF-κB signaling pathways compared to unstimulated cells. Although the DRY mutation has been until now considered to render US28 devoid of signaling *in vitro* (Bakker, Casarosa et al. 2004; Maussang, Verzijl et al. 2006), the signaling assays used may have hampered the identification of any activity for this receptor. Also, the situation in vivo may be different. In our xenograft model, mouse chemokines may activate US28-$R^{129}A$, leading to a continuous signaling activity, which, even if very low, may in the long term favor proliferation and lead to the formation of tumors. Also, NIH-3T3 cells possess chemokine receptors and functionally respond to CCL2 or CCL5 stimulation ((Hartmann, Leick et al. 2008) and Maussang et al., unpublished observations). Thus, in vivo, the murine chemokine-induced signaling of chemokine receptor present on stably transfected cells may cooperate with the US28-$R^{129}A$ receptor, resulting in the activation of transcription factors and subsequent tumor formation.

## Can G-protein independent signaling networks be activated by US28?

Human GPCRs signaling and cell surface expression are regulated by G protein regulatory kinases (GRKs). Typically, upon ligand stimulation, the activated receptor gets phosphorylated by GRK, which induces the recruitment of β-arrestins to the receptor, hindering G protein coupling. This results in a loss of signal transduction accompanied by endocytosis of the receptor (Kohout and Lefkowitz 2003). Besides desentisizing GPCRs, β-arrestins are also essential components of signaling networks (Lefkowitz and Shenoy 2005). Several studies have provided compelling evidence that β-arrestins mediate the activation of p42/p44 MAPK for a wide range of receptors, amongst others the angiotensin receptor $AT_{1A}R$ (Wei, Ahn et al. 2003), the CCR5, CCR7 and CXCR4 chemokine receptors (Sun, Cheng et al. 2002; Kohout, Nicholas et al. 2004), and the protease activated receptor-2 (PAR-2) (Ge, Ly et al. 2003). Other kinases from the p38 MAPK and c-Jun N-terminal kinase (JNK) families also use β-arrestins for their activation (Shenoy and Lefkowitz 2003). Mechanistically, arrestins directly bind to kinases and their regulatory effectors, forming signaling scaffolds that activate MAPK pathways (Shenoy and Lefkowitz 2003; Lefkowitz and Shenoy 2005), which have been extensively linked to chemotaxis and cell migration (DeFea 2007). For instance, lymphocytes derived from β-arrestin2 knock-out mice

presented reduced migration abilities towards CXCL12 (Fong, Premont et al. 2002), which may have been due to the impaired β-arrestin2-mediated activation of p38 MAPK by CXCR4 (Sun, Cheng et al. 2002). Similarly, cellular migration mediated through the protease-activated receptor-2 (PAR-2) (Ge, Ly et al. 2003) and the angiotensin II $AT_{1A}$ receptor (Hunton, Barnes et al. 2005) appears to rely on β-arrestin2. Recently, it was shown that β-arrestins can also mediate NF-κB transcriptional activation (Sun and Lin 2008). Stimulation of mouse embryonic fibroblasts (MEFs) with lysophosphatidic acid (LPA) resulted in activation of the NF-κB transcription factor and the release of IL-6, while MEFs deprived from β-arrestin2 failed to show such a phenotype. Mechanistically, LPA stimulation induces the recruitment of the scaffold protein CARMA3 to β-arrestin2, leading to NF-κB transcriptional activation and subsequent transcription of IL-6 (Sun and Lin 2008). Since β-arrestins are involved in the signaling of the PAR-2 receptor (Ge, Ly et al. 2003), a GPCR involved in carcinogenesis, these scaffold proteins may also play a role in the proliferation of cancer cells and the development of metastasis (Soreide, Janssen et al. 2006).

The different functions of β-arrestins may as such be involved in the tumorigenic potential of the US28-$R^{129}$A mutant observed in our xenograft model since they induce G protein-independent signaling. An important study by Wei et al. showed that mutation in the DRY motif of the $AT_{1A}$R receptor did not impair Ang II-induced p42/p44 MAPK activation (Wei, Ahn et al. 2003). Instead of using canonical G protein-mediated pathway, the G protein-uncoupled receptor was signaling via β-arrestin2 and this mechanism may also occur for the US28-$R^{129}$A mutant. US28-WT receptor has been shown to recruit β-arrestin2 to the cellular surface to mediate its signaling (Miller, Houtz et al. 2003). Since both US28-WT and US28-$R^{129}$A localize and internalize in similar fashions (Waldhoer, Casarosa et al. 2003), they may also recruit β-arrestins in similar manners. Thus, ligand stimulation of US28-$R^{129}$A may contribute to tumor formation via β-arrestin-dependent pathways. There is still very limited information on the signaling of US28-$R^{129}$A upon ligand stimulation, but one should keep in mind that the contributions of human and also mouse chemokine ligands need to be studied to explain the oncogenic phenotype observed in our xenograft model. In fact, US28 binds to murine CCL2 with a higher affinity than to human CCL2 (Maussang, unpublished observation). Additional studies will be required to elucidate the possibility and potential importance of G-protein independent signaling in US28-induced tumorigenesis.

### Can US28 transactivate other receptors in a G-protein independent manner?

Another "non-canonical" signaling ability of GPCRs resides in the transactivation of RTKs. Initially, it was found that stimulation of agonist ligands of endogenous GPCRs resulted in the phosphorylation of the epidermal growth factor (EGFR), which in turn mediated the activation of MAPK signaling pathways and cellular proliferation (Daub, Weiss et al. 1996). Several studies have now extended the transactivation mechanism to other RTKs such as the platelet-derived growth factor (PDGF) receptor (PDGFR) and fibroblast growth factor (FGF) receptor (FGFR) (Delcourt, Bockaert et al. 2007). Transactivation occurs according to two different mechanisms. Ligand-stimulated GPCR can activate downstream signaling pathways, e.g. production of reactive oxygen species, which in turn inhibit the activity of protein tyrosine phosphatases (PTP). Unable to dephosphorylate activated RTKs, PTPs no longer restrain RTK signaling (Werry, Sexton et al. 2005). A more extensively described transactivation mechanism resides in the GPCR-mediated activation of metalloproteases from the ADAM family, resulting in the shedding of surface-bound RTK ligands (Ohtsu, Dempsey et al. 2006). The activation of $G\alpha$ and $G\beta\gamma$ subunits as well as second messengers can activate ADAM, leading to the release of e.g. EGFR ligands (Ohtsu, Dempsey et al. 2006). This G protein-mediated transactivation of RTKs may occur with the constitutively active US28 receptor, but not with US28-$R^{129}$A. Interestingly, it was recently demonstrated that $\beta$-arrestins can also mediate EGFR transactivation independently from G proteins (Noma, Lemaire et al. 2007). Agonist stimulation of the $\beta$-adrenergic receptor $\beta_1$AR resulted in its phosphorylation by GRK and the subsequent recruitment of $\beta$-arrestin2 and Src. This led to matrix metalloproteinase activation, release of heparin-bound epidermal growth factor (HB-EGF) and transactivation of EGFRs (Noma, Lemaire et al. 2007). This could represent another alternative G protein-independent signaling pathway activated by US28-$R^{129}$A to induce signaling and tumor formation in vivo.

## IV. Molecular mechanisms of US28-induced oncogenesis

Our different studies of US28-induced proliferative and tumorigenic phenotypes in NIH-3T3 cells shed new light on molecular mechanisms related to the progression or development of proliferative diseases. Although we solely focused on COX-2 during the microarray analysis of

US28-expressing NIH-3T3 cells, several genes highlight other/new mechanisms used by US28 to induce tumorigenesis and further investigation will be necessary to evaluate the importance of such events.

## US28 and inflammation

Cancer has been epidemiologically related to chronic inflammation and it is believed that inflammation plays an important role in the development of proliferative diseases (Coussens and Werb 2002). The first inflammatory event demonstrated for US28 was the constitutive activation of NF-κB (Casarosa, Bakker et al. 2001). In **chapter 4** of this thesis, we also demonstrate that downstream of this transcription factor, COX-2 expression is constitutively upregulated in US28-expressing cells (Maussang, Langemeijer et al. 2009). This enzyme has been widely linked to inflammation and its expression has been reported to be upregulated in various forms of cancer (Turini and DuBois 2002). We also demonstrate here that COX-2 is an important component in the formation of US28-derived tumors and that its inhibition with Celecoxib can impair tumor formation and growth (Maussang, Langemeijer et al. 2009). In addition, **chapter 5** describes the US28-induced secretion of the pro-inflammatory cytokine IL-6. Constitutive NF-κB activation induces the transcription of IL-6 that can initiate a positive feedback loop with the STAT3 transcription factor. This positive feedback loop is further responsible for the pro-angiogenic and proliferative phenotypes of US28 stably transfected NIH-3T3 cells (Slinger, Maussang et al. In preparation). This increased IL-6 production correlates with the higher levels measured in the serum of patients with glioblastoma, colon and breast cancers (Hong, Angelo et al. 2007).

Furthermore, microarray analysis of US28-expressing NIH-3T3 cells indicates upregulation of Csf1, also known as M-CSF. Levels of this macrophage-specific stimulation growth factor are increased in many malignancies and correlate with poor prognosis (Hamilton 2008). CSF-1 leads to the recruitment of tumor-associated macrophages and the subsequent increased tumor growth (Lin, Nguyen et al. 2001; Mantovani, Marchesi et al. 2008). Our microarray analysis revealed that US28 also downmodulates anti-inflammatory proteins, such as the recently identified Edil3/Del-1 gene. Using a lipopolysaccharide (LPS)-induced lung inflammation model, Choi et al demonstrated that Edil3/Del-1 deletion significantly enhanced inflammation by increasing accumulation of neutrophils (Choi, Chavakis et al. 2008). Another anti-inflammatory component downregulated by US28 is Timp3. This enzyme is a natural inhibitor of tumor necrosis factor α-converting enzyme and

its genetic deletion in macrophages increases levels of secreted tumor necrosis factor $\alpha$. Also, Timp3$^{-/-}$ mice present a more severe LPS-induced systemic inflammation (Smookler, Mohammed et al. 2006). Thus, US28 may be able to induce a locally inflamed microenvironment to promote/enhance tumor growth.

## Regulation of oncogenes and tumor suppressors

When we compared the list of genes significantly modulated in US28-expressing NIH-3T3 cells to a database of genes involved in cancer (Maussang, Langemeijer et al. 2009), we identified several oncogene homologues and fusion proteins that may be involved in US28-mediated oncogenesis. One of the most upregulated cancer-related gene was Fus, which is a fusion gene initially discovered in liposarcoma (Crozat, Aman et al. 1993). Fus is highly upregulated in gastric carcinoma and it was postulated to be a potential biomarker for this proliferative condition (Oue, Hamai et al. 2004). In addition, the kinase Akt1, discovered as the oncogene transduced by the retrovirus AKT8 (Bellacosa, Testa et al. 1991), also seemed highly upregulated by US28. This kinase has been extensively linked to proliferative diseases and several TG animal models have confirmed its oncogenic potential (Bellacosa, Testa et al. 2004; Yang, Tschopp et al. 2004). Similar to various human cancers, US28-expressing NIH-3T3 cells also presented overexpression of Mdm2, which is a negative regulator of the tumor suppressor protein p53 (Onel and Cordon-Cardo 2004). Besides silencing p53, Mdm2 upregulation also promotes cell cycle progression (Martin, Trouche et al. 1995). In addition, the oncogenic potential of US28 may be attributed to the downregulation of several tumor-suppressor genes such as Fst, Rgs4 and Hivep3. Overexpression or downregulation of these molecules in cell lines have demonstrated their anti-proliferative potential (Allen and Wu 2000; Albig and Schiemann 2005; Chan, Ngan et al. 2009). Fst expression has also been shown to be downregulated in tissues of endometrical and cervical cancers (Chan, Ngan et al. 2009).

## US28 activates a wide selection of transcription factors

The constitutive activity of US28 was initially demonstrated using NF-$\kappa$B reporter gene assays in COS-7 transfected cells (Casarosa, Bakker et al. 2001). In addition, US28 also constitutively activated the CREB, NFAT and STAT3 transcription factors *in vitro* in HEK 293T cells (McLean, Holst et al. 2004; Slinger, Maussang et al. In preparation). STAT3 and CREB have been widely shown to play a role in oncogenesis and transformation (Siu and Jin 2007; Yu, Kortylewski et al. 2007). As

previously discussed, the role of NF-κB activation in (pre)-transformed and cancer cells is a crucial determinant for the induction of proinflammatory and prosurvival effectors (Perkins 2004). NFAT transcription factor was initially identified as regulator of gene expression in activated T cells (Shaw, Utz et al. 1988) and studies have highlighted the involvement of this transcription factor in oncogenic processes such as COX-2-mediated cancer cell invasion (Yiu and Toker 2006). However, a recent study highlighted that different members of the NFAT family may possess different involvement in oncogenic situations. Robbs et al., demonstrated that in NIH-3T3 cells, overexpression of NFAT1 induced apoptosis while NFAT2 mediated cellular proliferation and tumorigenesis in vivo (Robbs, Cruz et al. 2008). As such, the constitutive activation of NFAT by US28 may deserve a more detailed investigation to determine which subtypes may contribute to viral oncogenesis, and potentially apoptosis. In NIH-3T3 cells, US28-mediated NFAT constitutive activation may be linked to the upregulation of Rcan2 mRNA. This gene is a transcriptional target of NFAT that acts as a negative regulator of NFAT-mediated transcription (Gollogly, Ryeom et al. 2007). Interestingly, other new transcription factors may be constitutively induced in stably transfected NIH-3T3 cells. For instance, the Cebpb transcription factor mRNA was highly upregulated. This result reflects the overexpression observed in certain cancers such as the anaplastic large cell lymphoma (Piva, Pellegrino et al. 2006). Although Cebpb presents tumor-suppressor activities in mouse embryo fibroblasts, increasing evidence suggests that this transcription factor is involved in oncogenic processes (Sebastian and Johnson 2006). Interestingly, the upregulation of Cebpb may be a consequence of the US28-induced IL-6 secretion and STAT3 activation in NIH-3T3 cells (Slinger, Maussang et al. In preparation). Cebpb, also known as nuclear factor of interleukin 6, is transcriptionally activated in IL-6 (Kuilman, Michaloglou et al. 2008) and STAT3 (Piva, Pellegrino et al. 2006) dependent manners in different oncogenic settings. Finally, the Mef2c transcription factor is upregulated on the mRNA level in US28-expressing NIH-3T3 cells. This does not demonstrate transcriptional activation of Mef2c per se, but the concomitant upregulation of Hdac9 (Haberland, Arnold et al. 2007) suggests that US28 also activates Mef2c in NIH-3T3 cells. This transcription factor is involved in the proliferation of different cell types such as B cells (Khiem, Cyster et al. 2008) and vascular smooth muscle cells (Zhao, Liu et al. 2002). Mef2c may also plays a role in the development of gastric (Ohta, Aoyagi et al. 2009) and breast cancers (Ostrander, Daniel et al. 2007). Transcriptional regulation is not only mediated by classical transcription factors, but it can also be induced by nuclear receptors. US28-expressing cells show increased expression of Nr4a2 mRNA expression. This nuclear receptor mediates

the proliferative and apoptosis-resistant phenotype observed in cervical cancer HeLa cells (Ke, Claassen et al. 2004) and is transcriptionally activated by the COX-2-derived prostaglandin PGE2 in colorectal cancer cells (Holla, Mann et al. 2006). As such, this upregulated gene may result from COX-2 overexpression in US28-expressing NIH-3T3 cells.

## V. US28 role in HCMV oncomodulation

### NF-κB and cellular context: a clue towards US28 role in HCMV oncomodulation?

HCMV is not considered an oncogenic virus but its ability to preferentially infect cancer cells has resulted in the hypothesis that this virus is able to enhance the oncogenic potential of transformed cells (Michaelis, Doerr et al. 2009). As such, the genetic background of the infected cell may exacerbate the ability of HCMV to enhance oncogenic development. Interestingly, similar observations occur for NF-κB cellular functions. Data suggest that transcriptional activity of NF-κB in untransformed cells activates tumor suppressor programs (Perkins and Gilmore 2006). However, as the cell accumulates an oncogenic potential, genetic mutations may lead to the loss of tumor-suppressing genes resulting in the tumor-promoting activity of NF-κB (Perkins 2004). Because of the similar pro-oncogenic abilities of HCMV and NF-κB in (pre)-cancer cells, these two factors may be intertwined. In fact, HCMV infection is already known to induce NF-κB activation (Kowalik, Wing et al. 1993), and we now have demonstrated that this signaling is mediated at least by US28 (**chapter 4**). As such, the constitutive activation of NF-κB by US28 in HCMV-infected cancer cells would in turn mediate transcription of tumor-enhancing factors and increase the malignancy of the transformed cell.

**Table 2.** Molecular effectors of US28-induced tumorigenesis as detected in the microarray analysis of US28-expressing NIH-3T3 cells or with *in vitro* assays

| Gene | Modulation | Expression and/or associated function | Reference |
|---|---|---|---|
| *Inflammatory factors* | | | |
| Cox-2 | Up | Pro-inflammatory enzyme and overexpressed in a variety of cancers | Turini and DuBois 2002 |
| Csf1 | Up | Increased levels in many malignancies leading to macrophage recruitment and proliferation, and subsequent increased tumor growth | Hamilton 2008; Mantovani, Marchesi et al. 2008 |
| Edil3/Del-1 | Down | Anti-inflammatory function demonstrated in Del-1-/- mice. Genetic deletion exacerbates LPS-induced inflammation | Choi, Chavakis et al. 2008 |
| Timp3 | Down | Genetic deletion leads to increased secretion of inflammatory factor TNFa in vitro and enhanced inflammatory response in vivo | Smookler, Mohammed et al. 2006 |
| Il-6 | Up | Upregulated in various types of cancer and mediates cellular proliferation and immune suppression | Hong, Angelo et al. 2007; Yu, Kortylewski et al. 2007 |
| | | Induces reccurent inflammation in patients with inflammatory bowel disease | Mitsuyama, Sata et al. 2006 |
| *Oncogenes and tumor suppressors* | | | |
| Fus | Up | Upregulated in gastric carcinoma | Oue, Hamai et al. 2004 |
| Akt1 | Up | Upregulated in esophageal squamous cell carcinoma | Takahashi, Miyashita et al. 2008 |
| | | Usually Akt2 upregulated in various cancers | Bellacosa, Testa et al. 2004 |
| | | Role in proliferation investigated in vivo with TG mice: link to proliferation | Yang, Tschopp et al. 2004 |
| Mdm2 | Up | Up in various cancers. Poor prognosis in sarcoma and glioma | Onel and Cordon-Cardo 2004 |
| | | Promotes cell cycle progression | Martin, Trouche et al. 1995 |
| Fst | Down | Downregulated in endometrial and cervical cancers. Overexpression in vitro reduces cell migration and invasion. | Chan, Ngan et al. 2009 |
| Rgs4 | Down | Overexpression delays tubulation of epithelial cells and p38 activation. RGS4 also inhibits VEGF-induced signaling by decreasing VEGF receptor-2 expression | Albig and Schiemann 2005 |
| Hivep3 | Down | Downregulation promotes cell cycle progression in HeLa cells | Allen and Wu 2000 |
| *Transcription factor* | | | |
| CREB | Activated | Overexpressed or constitutively activated in various forms of cancer | Siu and Jin 2007 |
| STAT3 | Activated | Constitutively activated in cancer cells | Yu, Kortylewski et al. 2007 |
| NF-kB | Activated | Tumor promoting activity in cancer cells | Perkins 2004 |
| NFAT | Activated | Different isoforms of NFAT act as tumor-inducer or -suppressor in NIH-3T3 cells | Robbs, Cruz et al. 2008 |
| Cebpb | Up | Involved in certain types of cancer and activated in IL-6 and STAT3 dependent manners | Sebastian and Johnson 2006; Kuilman, Michaloglou et al. 2008; Piva, Pellegrino et al. 2006 |
| Rcan2 | Up | Transcriptionally activated by NFAT and negative regulator of NFAT | Gollogly, Ryeom et al. 2007 |
| Mef2c | Up | Upregulates Hdac9 mRNA transcription | Haberland, Arnold et al. 2007 |
| | | May be involved in gastric anc breast cancer | Ohta, Aoyagi et al. 2009; Ostrander, Daniel et al. 2007 |
| Nr4a2 | Up | Induced by COX-2-derived PGE2 and mediates proliferation and resistance to apoptosis | Ke, Claassen et al. 2004; Holla, Mann et al. 2006 |

## STAT3 activation and immune suppression

The development of malignancies is not only due to the growth of cancer cells, but it is also intrinsically linked to the immune system. Various studies document the role of the immune system during tumor surveillance and elimination (reviewed in (Dunn, Bruce et al. 2002)). Cells from the innate immune system recognize transformed cells and initiate responses that will lead to the secretion of angiostatic and anti-proliferative factors leading to inhibition of tumor vascularization and growth. Other immune effector cells migrate towards the tumor sites by sensing and following chemokine gradients. This immune response results in turn in the destruction of tumor cells (Dunn, Bruce et al. 2002). Interestingly, transformed cells are able to produce soluble proteins (e.g. IL-6, VEGF) and activate transcription factors (e.g. STAT3) that hinder immune surveillance (Yu, Kortylewski et al. 2007). Constitutive or ligand (e.g. IL-6)-induced activation of STAT3 induces secretion of factors, such as VEGF, IL-6 and IL-10, which inhibit the functions of immune cells. For instance, STAT3 activation in dendritic cells impairs their maturation and ability to present antigens to CD4+ T cell, resulting in the degeneration of T-cell mediated immune responses. In addition, STAT3 activation in regulatory T cells infiltrated within tumor sites induces their proliferation and the secretion of TGFβ and IL-10. These factors subsequently suppress CD8+ T cell activation and related cytotoxic activity (Yu, Kortylewski et al. 2007). The generation of such an immunosuppressed environment by tumor cells seems ideal for HCMV infection. In addition, cancer cells usually present overexpression of RTKs such as EGFR and PDGFR, which are important factors for viral entry and cellular infection (Kim 2004) (Roussidis, Theocharis et al. 2007). Thus, cancer cells provide a safe niche for HCMV to replicate and further enhance its pathological activities. Upon infection of transformed cells, the expression of US28 and the constitutive activation of STAT3 may further exacerbate locally suppressed immune responses and allow the virus to aggravate the development of malignancies.

## HCMV and colon cancer

HCMV infection has been linked to the development of inflammatory bowel diseases such as Crohn's disease and inflammatory colitis, which is a common HCMV-derived diseases in immunocompromosed patients (Britt 2008). Patients presenting colitis are also more prone to develop colon cancer, which may explain the high prevalence of HCMV in such cancer patients (Harkins, Volk et al. 2002). Protein and DNA products of HCMV were detected in various tissues from colitis patients (Rahbar, Bostrom et al. 2003). Gastrointestinal proliferative diseases usually are

mediated by an inflammatory component and pro-inflammatory factors are known to play significant roles in this process. For instance, cyclooxygenase-2 (COX-2) upregulation has been widely reported in colorectal cancer and numerous studies investigated its inhibition in this context (Gupta and Dubois 2001). Colon cancer patients present aberrantly elevated COX-2 expression and PGE2 secretion levels, and COX-2 inhibitors can reduce disease progression (Backlund, Mann et al. 2005). COX-2 is responsible for the synthesis of prostaglandin PGE2, which we have shown to be induced by US28 in HCMV-infected cells (Maussang, Langemeijer et al. 2009). PGE2 is an important mediator of cellular proliferation and sustained angiogenesis. In particular, the binding of PGE2 to its cognate receptor EP2 activates several transcription factors such as CREB, Tcf-Lef and AP1 and induces the transcription of proliferative and angiogenic factors (Castellone, Teramoto et al. 2005; Dorsam and Gutkind 2007). Amongst those, Cyclin D1, Nr4a2 mediate cellular proliferation and resistance to apoptosis, while VEGF gene expression favors angiogenesis. In addition to COX-2 and PGE2, IL-6 may be a crucial component in the promotion of inflammation. Patients with IBD and colon cancer present increased levels of IL-6 and this cytokine is suggested to perpetuate inflammation and mediate cellular proliferation (Mitsuyama, Sata et al. 2006). The anti-apoptotic function of IL-6 was shown by injecting stably-transfected CHO cells secreting IL-6 into nude mice (Jin, Zimmers et al. 2008). This allowed intestinal villi and the entire small intestine to present an increased length. In this context, IL-6 was presented as an anti-apoptotic rather than a proliferative cytokine. However, two recent studies also highlighted the importance of the NF-κB/IL-6/STAT3 axis in colon oncogenesis (Bromberg and Wang 2009). IL-6 secretion induced in an azoxymethane (AOM) carcinogenesis model resulted in STAT3 activation in intestinal epithelial cells (IEC) and their subsequent proliferation and decreased sensitivity to apoptosis (Grivennikov, Karin et al. 2009). In addition, loss- or gain-of-function of STAT3 in intestinal epithelial cells confirmed that STAT3 is an important mediator of cellular proliferation and anti-apoptotic activity in a colitis-associated colon cancer model (Bollrath, Phesse et al. 2009). Since HCMV early antigens were detected together with IL-6 in a great majority of tissues from ulcerative colitis patient (Rahbar, Bostrom et al. 2003), this may provide a crucial role for US28 in this oncogenic/inflammatory process. One could hypothesize that US28 is expressed upon viral infection of cancer cells and induces the constitutive activation of STAT3 and COX-2. This would subsequently lead to the release of IL-6 and PGE2. These factors, in an autocrine or paracrine manner, can in turn potentiate inflammation, induce proliferation and further protect cancer cells from apoptosis.

### US28 and glioblastoma development

HCMV infection is increasingly linked to the development of glioblastoma (Miller 2009). A high proportion of malignant glioma are infected with HCMV (Cobbs, Harkins et al. 2002). This finding was recently confirmed and HCMV infection was correlated with the disease stage (Scheurer, Bondy et al. 2008). COX-2 and IL-6 levels are elevated in glioblastoma patients and seem to be associated with tumor grade (Tchirkov, Khalil et al. 2007; Chuang, Kardosh et al. 2008). The therapeutic potential of specific COX-2 inhibition did not seem as promising as in the case of colon cancer but a more general anti-inflammatory approach may be a better approach (Chuang, Kardosh et al. 2008). Until now, IL-6 and STAT3 are regarded as potential targets for the treatment of this invasive disease. The importance of IL-6 in glioblastoma pathology was also supported through increased expression of its transcription factor Cebpb in glioma patients. Similarly to IL-6, Cebpb expression correlated with negative disease outcome (Homma, Yamanaka et al. 2006). STAT3 overactivation has also been documented in glioblastomas and presents significant therapeutical potential (Brantley and Benveniste 2008). As such, the activation of the IL-6/STAT3 pathway by US28 (Slinger, Maussang et al. In preparation) could represent a molecular link between HCMV infection and glioblastoma development. By regulating US28 expression in these brain tumors, HCMV may enhance proliferation, angiogenesis and tumor evasion to worsen disease progression.

## VI. Future perspectives

### The HCMV-encoded chemokine receptor US28

The definition and description of the so-called "oncomodulation" induced by HCMV is a very interesting concept proposed by Cinatl et al. (Cinatl, Cinatl et al. 1996). The observation that HCMV does not transform cells but rather enhances their oncogenic potential is something which also seems to apply to the viral chemokine receptor US28. Our observation that US28 presents oncogenic properties in NIH-3T3 cells is very exciting but still remains intriguing. Due to the observed US28-induced apoptosis in HEK 293T cells and the difficulty to obtain stable cell lines expressing this viral chemokine receptor, the transforming ability of US28 may necessitate a favorable genetic background. Cells possessing oncogenic properties, i.e. with oncogenic mutations and altered cellular gene regulations, may be a prerequisite for US28 to exert its oncogenic

potential. As such, the oncomodulation concept needs to be kept in mind for future studies of US28 in a viral context or in cell lines *in vitro*. The expression of US28 in various cell lines and the determination of the activated molecular effectors could reveal important factors involved in the balance between apoptosis and proliferation. It will be particularly relevant to investigate the role of US28 in glioblastoma and colon cancer cells since both diseases are increasingly recognized as HCMV-related diseases. Molecular mechanisms governing oncomodulation in these cells may identify US28 or downstream effectors as potential therapeutical targets for the treatment of viral diseases. In addition, the study of the paracrine effect induced by expression of US28 still remains of interest. Since cancer development is intertwined with chronic inflammation (Coussens and Werb 2002) and since US28 induces a pro-inflammatory phenotype, it will be crucial to investigate the paracrine effect of US28-expressing or HCMV-infected cells on other cell types, e.g. uninfected cells or cells from the immune system. Also, as exemplified for the KSHV-encoded chemokine receptor ORF74, transformation induced by paracrine mediators may be an important component in US28-mediated pathologies.

Beside *in vitro* studies, the generation of transgenic animals expressing US28 will be a very valuable tool to understand the importance of this vGPCR in HCMV-related diseases. Regarding the concept of HCMV oncomodulation and the favorable "infectable niche" of immunosuppressed and genetically unstable tumor cells, the transforming ability of US28 may also be apparent only in context. For instance, pathological conditions such as colon cancer and glioblastoma present overactivated proliferative signaling pathways, e.g. NF-κB, COX-2, VEGF, STAT3, which could further be exacerbated with the expressions of the constitutively active US28 receptor. Cells may require a certain oncogenic or signaling threshold that US28 may boost to promote transformation. As such, US28 function may be studied in the context of mice models of pathologies related to HCMV infection. For instance, US28-expressing mice may be related to $APC^{-/-}$ mice for the development of colon cancer (Taketo 2006) or glioblastoma mouse models obtained by lentiviral delivery of oncogenes (Marumoto, Tashiro et al. 2009). In a similar manner, the use of atherosclerosis mouse models, such as $ApoE^{-/-}$ mice (Zadelaar, Kleemann et al. 2007), may allow us to understand the role of US28 in vivo during the development of cardiovascular diseases.

## Other CMV-encoded vGPCR

US28 has been extensively studied and linked to several pathological conditions. However, the presence of other vGPCRs in the HCMV genome implies that those may also be important in various viral processes. In particular, UL33 genes are conserved amongst herpesviruses, and UL33 proteins from the human, mouse and rat CMV are constitutively active (Gruijthuijsen, Casarosa et al. 2002; Waldhoer, Kledal et al. 2002). Interestingly, in HCMV-infected cells, UL33 is responsible for the constitutive CREB-mediated gene transcription, while US28 constitutively induces inositol phosphate formation. Thus, the constitutively active vGPCR UL33 and US28 may play different roles in infected cells, but they may also present overlapping functions. In fact, US28 is not present in mouse and rat CMV, and it is not conserved in all CMV species, while UL33 is. Mouse and rat CMV encode UL33 genes that are able to induce cellular migration, mimicking the described chemotactic function of the HCMV-encoded US28 gene (Streblow, Soderberg-Naucler et al. 1999; Melnychuk, Smith et al. 2005; Streblow, Kreklywich et al. 2005). The M33 protein induces migration of mouse cells towards mCCL5, and deletion mutants of MCMV lacking M33, or RCMV missing the ORF R33 induce impaired cellular migration than their respective WT viruses (Melnychuk, Smith et al. 2005; Streblow, Kreklywich et al. 2005). Recently, it was shown that UL33 could compensate the lack of M33 in a MCMV deletion mutant, suggesting conserved biological functions of the UL33 gene family (Case, Sharp et al. 2008). Signaling studies in transfected cells and infected cells will determine what signaling pathways other than CREB activation are attributed to UL33, and how the receptor activity may be implicated in viral life cycle and/or viral pathogenesis.

The other genes conserved amongst CMV species belong to the UL78 family. Studies of rat and mouse CMV showed that the UL78 genes are important for viral replication (Oliveira and Shenk 2001; Kaptein, Beisser et al. 2003). However, deletion of the UL78 gene from the HCMV genome did not impair viral replication (Michel, Milotic et al. 2005). Since the UL78 protein is not constitutively active and its ligands are still unknown, signaling studies seem premature at this point. In a similar manner, the HCMV-encoded US27 gene (which is not present in mouse and rat CMV) has not shown constitutive signaling and its role for HCMV remains puzzling. Deorphanization of both US27 and UL78 genes is a first step that will allow characterization of the signaling properties of these receptors. In addition, similarly to the EBV-encoded vGPCR BILF1, the UL78 and US27 proteins may be able to heterodimerize with other cellular receptors to modulate cellular signaling pathways (Vischer, Nijmeijer et al. 2008).

**Gamma herpesviruses: EBV and KSHV**

Both γ1- (EBV) and γ2-HHV (KSHV) are known oncogenic viruses that have been extensively linked to the development of lymphoproliferative diseases. They both encode a single vGPCR, namely BILF1 and ORF74, which in both cases exhibit constitutive activity. ORF74 activates a broad range of G proteins, such as $G\alpha_q$, $G\alpha_{12/13}$, $G\alpha_{i/o}$ and $G\alpha_s$, and also downstream kinases, subsequently resulting in cellular proliferation (Rosenkilde, Smit et al. 2008). In a different manner, the EBV-encoded BILF1 protein solely activates $G\alpha_i$ proteins (Beisser, Verzijl et al. 2005), which may already indicate why these γ-HHV-encoded vGPCR, BILF1 and ORF74, seem to present different functions. ORF74 is recognized as a viral oncogene and its expression induces the development of lesions resembling those observed in KSHV-infected patients. Several TG animal models highlighted the importance of this vGPCR in cellular transformation and oncogenesis, leading to the characterization of ORF74 as a viral oncogene. Interestingly, we show that the EBV-encoded BILF1 receptor presents a quite different function. Expression of BILF1 in the B cell compartment of TG mice results in B cell lymphopenia due to an increased apoptotic activity. This indicates that the function of the lytic gene BILF1 is to induce apoptosis. In addition, another function of the BILF1 gene, which is conserved amongst γ1-herpesviruses (Beisser, Verzijl et al. 2005), is to subvert immune surveillance. We had initially demonstrated that BILF1 constitutive signaling inhibits PKR activation (Beisser, Verzijl et al. 2005), impairing cellular antiviral responses. Zuo et al. also recently showed that BILF1 contributes to the immune escape of EBV-infected cells by downregulating MHC class I expression (Zuo, Currin et al. 2009). It would be interesting to investigate what is the kinetic of expression of BILF1 in EBV-infected cells during the lytic phase to determine if the apoptotic and immune evasion functions of BILF1 rely on its expression levels or kinetics. In addition, BILF1-expressing B cells derived from TG animals should be used to determine if PKR inhibition and MHC class I downregulation are also observed in vivo. Since EBV lytic infection, and presumably BILF1 expression, occurs in plasma B cells in infected human hosts (Laichalk and Thorley-Lawson 2005), it would be particularly relevant to study BILF1 phenotype in this specific B cell subpopulation. Also, the generation of transgenic animals expressing BILF1 specifically in plasma B cells would allow us to understand how BILF1 influences cellular fate and which mechanisms are activated during this process.

## Conclusion

Taken together, our data demonstrate that the constitutively active HCMV-encoded chemokine receptor US28 induces a pro-angiogenic and proliferative phenotype, leading to tumor formation in a xenograft model. Mechanistically, US28 induces a pro-inflammatory phenotype by activating various transcription factors, e.g. NF-κB and STAT3, further inducing the expression of pro-inflammatory factors such as COX-2 and IL-6. In HCMV-infected cells, US28 also constitutively activates several signaling pathways implicated in pathological conditions. As such, US28 represents a molecular link between viral infection and the development of proliferative diseases, which strengthens the interesting therapeutic potential of US28.

The generation of BILF1-expressing TG animals indicates that the EBV-encoded vGPCR induces apoptosis in vivo. The pro-apoptotic role of BILF1 differs from the oncogenic potential of the KSHV-encoded ORF74 gene. These data implicate that these two different viral gene families have different functions during either lytic viral replication or viral pathogenesis.

# VIII

Nederlandse Samenvatting

**Herpesvirus-gecodeerde G-eiwit-gekoppelde receptoren als modulatoren van cellulaire signalering**

Humane Herpesvirusen (HHV) zijn alomvertegenwoordigd in de bevolking. Meestal zijn ze onschadelijk na eerste infectie, maar ze hebben de capaciteit om latent aanwezig te blijven in de geïnfecteerde gastheer. Bij slecht functioneren of tijdelijke onderdrukking van het immuunsysteem van de gastheer worden herpesvirussen geheractiveerd, wat tot sterke pathologische omstandigheden kan leiden.

HHV-5, ook bekend als humaan cytomegalovirus (HCMV), is een belangrijke ziekteverwekker in transplantatiepatiënten waarbij het immuunsysteem is onderdrukt en lijkt betrokken te zijn bij de ontwikkeling van vasculaire en proliferatieve ziekten (Soderberg-Naucler 2006). Een ander veelonderzocht herpesvirus is HHV-4, vooral bekend onder de naam Epstein-Barr Virus (EBV), welke betrokken is bij proliferative ziekten van de lymfocyten zoals Hodgkin- en Burkittlymfoom (Kutok and Wang 2006).

Herpesvirussen hebben gedurende hun evolutie meerdere manieren ontwikkeld om het immuunsyteem van de gastheer te beïnvloeden, om zodoende stand te houden. Ze hebben genen van de gastheer geïntegreerd in hun genoom en deze aangepast in hun eigen voordeel. HHVs van de beta en gamma families bezitten genen die coderen voor virale G eiwit gekoppelde receptoren (vGPCRs). Deze stammen af van de menselijke chemokine receptoren. Bijvoorbeeld de genomen van HCMV en EBV bevatten respectievelijk vier (US27, US28, UL33, UL78) GPCRs en één (BILF1) GPCR (Rosenkilde, Smit et al. 2008). De karakteristieken van het grootste deel van deze vGPCRs zijn zodanig dat ze, in tegenstelling tot humane chemokine receptoren, op een constitutieve manier kunnen signaleren. Dit heeft de modulatie van de intracellulaire signaleringsroutes tot gevolg en leidt tot diepgaande effecten op de het biologische gedrag van de getroffen cel. Een opvallend voorbeeld van het fysiologische belang van vGPCRs is aangetoond met het ORF74 gen van HHV-8 (ook bekend onder de naam Kaposi's sarcoma-associated herpesvirus, KSHV). Talrijke *in vitro* en *in vivo* studies hebben aangetoond dat de expressie van deze virale chemokine receptor leidt tot cellulaire veranderingen (Bais, Santomasso et al. 1998) en de ontwikkeling van Kaposi's sarcoma-achtige laesies in ORF74 tot expressie brengende transgene muizen (Yang, Chen et al. 2000). Dit laat zien dat de expressie van één enkele vGPCR van cruciaal belang is in KCHV- gerelateerde ziekten.

Het doel van dit proefschrift is het begrijpen van de biologische relevantie van de HCMV-gecodeerde chemokine receptor US28 en van

de EBV-gecodeerde vGPCR BILF1, met andere woorden, zijn deze eiwitten betrokken bij de levenscyslus van het virus en/of virale ziektebeelden?

Hoofdstuk 1 en 2 zijn introductiehoofdstukken die uitgebreid de huidige wetenschappelijke kennis van vGPCRs beschrijven. Hoofdstuk 1 in het bijzonder beschrijft de algemene kennis van alle bekende HHVs van de beta en gamma subfamilies, door het geven van achtergrondinformatie over de structuur van deze virussen, viraliteit, virale routes, maar ook de ziektes waaraan ze gerelateerd zijn. Daarnaast beschrijft dit hoofdstuk alle vGPCRs van deze virussen en hun link met de pathologische processen en de geassocieerde virale ziekten. Hoofdstuk 2 richt zich op de verschillende technieken die gebruikt worden om deze vGPCRs te bestuderen. Uitgaande van de technieken die wij gebruiken in ons onderzoek, wordt een breed scala aan protocollen gegeven, variërend van moleculaire biologie naar celbiologie, virologie en oncologie.

Hoofdstukken 3 tot en met 5 richten zich op de HCMV gecodeerde chemokine receptor US28. In hoofdstuk 3 laten we het tumorvormende vermogen van US28 zien. Wanneer US28 stabiel tot expressie wordt gebracht in NIH-3T3 cellen, kunnen we laten zien dat deze receptor op een constitutieve manier cellulaire proliferatie en de secretie van de angiogenese factor VEGF (vasculaire endotheliale groeifactor) kan induceren. Studies gebruik makend van transiente transfecties wijzen kinases en transcriptie factoren aan die de expressie van VEGF reguleren in US28 tot overexpressie brengende COS-7 cellen. Ten slotte leidt xenograft implantatie van NIH-3T3 cellen die US28 tot expressie brengen tot de vorming van tumoren in naakte muizen. Om te onderzoeken welke oncogenese factoren verantwoordelijk zijn voor de capaciteit van US28 om cellen te veranderen, gebruikten we een microarray-aanpak in hoofdstuk 4 en tonen we aan dat het kanker-gerelateerde enzym cyclooxygenase 2 (COX-2) een belangrijke factor in US28-gemedieerde transformatie is. Met behulp van een COX-2 specifieke remmer, Celecoxib, kunnen we *in vitro* de uitscheiding van VEGF en *in vivo* de vorming van tumoren remmen. In hoofdstuk 5 gebruikten we een andere aanpak om te onderzoeken welke factoren US28 to expressie brengende NIH-3T3 cellen uitscheiden. We laten zien dat US28 naast VEGF ook interleukine (IL-)6 laat vrijkomen. Gelijk aan wat eerder is waargenomen in verscheidene vormen van kanker, tonen we aan dat US28 een positieve terugkoppeling tussen de IL-6 secretie en de activering van STAT3 (signaal transducer en activator van transcript 3) induceert. De activering van de IL-6/STAT3 as via de NF-κB transcriptie factor is van belang voor de waargenomen proliferatie van de US28 tot expressie brengende NIH-3T3 cellen. Zeer interessant is dat

experimenten in hoofdstuk 3 tot en met 5 ook laten zien dat met het verwijderen van het US28-gen uit het HCMV genoom de constitutieve activering van signaleringstrajecten in de besmette cellen vermindert. Cellen geïnfecteerd met deze HCMV US28 deletie mutant laten een verminderde activering van STAT3 en de promotorgenen van VEGF en COX-2 zien. De constitutieve activiteit van US28 in HCMV-geïnfecteerde humane glioblastoma cellen lijkt daarom een belangrijke rol te spelen in de virale activering van kanker-gerelateerde signaleringsroutes.

De rol van de door EBV-gecodeerde vGPCR BILF1 werd onderzocht in transgene muizen die dit eiwit tot expressie brengen in B-cellen. B-lymfocyten-specifieke expressie werd gekozen op basis van de tropisme van het virus voor deze cellulaire subpopulatie. Transgene muizen ontwikkelen verrassend genoeg een ernstige B cel lymfopenie, gekenmerkt door een afname in het totale aantal B-cellen in verschillende organen zoals beenmerg, milt en lymfeklieren. Deze B-cel-lymfopenie leidt tot een algemene daling van de titers van immunoglobulinen, hetgeen aanduidt dat deze muizen een slechter immuunsysteem hebben. Verder is het vermogen van transgene muizen om te reageren op antigenen gedaald in vergelijking met wild-type muizen. De B-cel lymfopenie blijkt te wijten te zijn aan een uitgebreide apoptose van de B-cellen, zoals aangetoond door middel van Annexine V kleuring.

Samenvattend laat dit proefschrift zien dat de consitutieve activiteit van de HCMV-gecodeerde vGPCR US28 verantwoordelijk is voor de ongecontroleerde proliferatie van cellen en een rol kan spelen in proliferatieve virale ziekten. Dit in tegenstelling tot de EBV vGPCR BILF1 die *in vivo* constitutief cellulaire apoptose lijkt te induceren en daarmee een functie kan hebben tijdens de lytische replicatie van het virus.

# IX

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# X

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Maussang, D., D. Verzijl, et al. (*2006*). "Human cytomegalovirus-encoded chemokine receptor US28 promotes tumorigenesis". Proc Natl Acad Sci U S A 103(35): 13068-73.

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Maussang, D., H. F. Vischer, et al. (*2009*). "Herpesvirus-encoded G protein-coupled receptors as modulators of cellular function". Mol Pharmacol In press.

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Maussang, D., G. C. Furtado, et al. (*In preparation*). "The Epstein Barr virus-encoded G protein-coupled receptor BILF1 induces B cell apoptosis *in vivo*".

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# XI

Final words

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